THE CONSTRUCTION OF PLANT EXPRESSION VECTORS FOR THE INTRODUCTION OF LEAFROLL DISEASE RESISTANCE IN GRAPEVINE

Celene Debra van Straten

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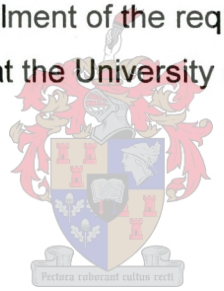

Johan T. Burger

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"Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

ABSTRACT

Grapevine leafroll is one of the most damaging viral diseases that affect many viticultural regions of the world. Numerous reports over the last few years have associated closterovirus-like particles with leafroll disease. To date, eight serologically distinct closteroviruses have been isolated from leafroll infected vines, of which grapevine leafroll associated closterovirus-3 (GLRaV-3) is the best characterized.

Virus resistance in transgenic plants based on the expression of a virus-derived gene is known as pathogen-derived resistance. The viral coat protein (CP) gene, which expresses a structural protein responsible for coating the virus particles, was used in the first demonstration of virus-derived resistance. Coat protein-mediated resistance is currently the most feasible and most widely used method to obtain virus resistance in crop plants.

The CP gene of a South African isolate of GLRaV-3 infected grapevine was isolated, cloned and sequenced. Double stranded RNA (dsRNA) was extracted from GLRaV-3 infected material and a high molecular weight band, of ~18 kb was identified from infected vines. The dsRNA was used as a template in a reverse transcription PCR together with GLRaV-3 CP gene specific primers for the amplification of the GLRaV-3 CP gene (975 bp). The GLRaV-3 CP gene was cloned into the pGem®-T Easy vector. Clones hosting the CP gene in the sense (pLR3CP+) and antisense (pLR3CP-) orientations respectively were obtained. The sequence obtained from these two clones showed 99.26 % similarity to the only other GLRaV-3 CP nucleotide sequence available. The GLRaV-3 CP gene was excised from pLR3CP+ and pLR3CP- and subcloned into a plant expression vector, pCAMBIA 3301 in the sense (pCamBLR3CP+) and antisense (pCamBLR3CP-) orientations respectively, therefore enabling sense and antisense gene expression in transgenic plants. The GLRaV-3 CP gene was also subcloned from pCamBLR3CP+ into another plant expression vector, pCAMBIA 2301 in the sense orientation and designated as pCVSLR3CP+. These three constructs were given to Dr. M. Vivier (Institute for Wine Biotechnology, Stellenbosch) for grapevine transformation experiments. Two of these constructs, pCamBLR3CP+ and pCamBLR3CP- as well as pCAMBIA

3301 were used to transform *Nicotiana tabacum* by *Agrobacterium tumefaciens*-mediated transformation. Plants were selected for their ability to withstand the herbicide, Basta. This resistance is due to the presence of a plant selectable marker gene on each of these constructs, known as the *bar* gene. PCR with GLRaV-3 CP gene specific primers showed no amplification of the GLRaV-3 CP gene in the plants transformed with pCamBLR3CP+ and pCamBLR3CP-. Southern blot analysis with the GLRaV-3 CP gene as hybridization probe showed no signal for these plants, thus confirming the PCR results. PCR with *bar* gene specific primers showed no amplification of the *bar* gene in the plants infected with pCAMBIA 3301. The plants transformed with pCamBLR3CP+ and pCamBLR3CP- were also screened for the presence of the *bar* gene. Three of the eight plants tested showed amplification of the ~560 bp *bar* gene. This result suggests that these plants were transformed with pCAMBIA 3301 (vector without the ligated GLRaV-3 CP gene) and not pCamBLR3CP+ or pCamBLR3CP- as had been expected. This project provides preliminary work for the subsequent transformation of grapevine with the GLRaV-3 CP gene, in an attempt to impart virus resistance.

OPSOMMING

Wingerd rolblaar is een van die mees beskadigende virale siektes wat baie wingerd areas in die wêreld aantas. 'n Aantal verslae oor die afgelope jare het closterovirus partikels met wingerd rolblaar geassosieer. Tot hede, is agt serologiese onderskeibare closterovirusse geïsoleer vanuit geaffekteerde wingerde, waarvan wingerd rolblaar geassosieerde closterovirus-3 (GLRaV-3) die beste gekarakteriseerd is.

Virus bestandheid in transgeniese plante gebaseer op die uitdrukking van gene afkomstig vanaf virusse, staan bekend as patogeen-afgeleide weerstand. Die virale kapsule protein (CP) geen vervaardig 'n strukturele protein wat verantwoordelik is vir die bedekking van die virus partikel. Dié geen was gebruik in die eerste demonstrasie van patogeen-afgeleide weerstand. Kapsuul protein-bemiddelde weerstand is tans die mees praktiese en algemene gebruikte metode om virus weerstand in plant gewasse te verkry. Die CP geen van 'n Suid Afrikaanse isolaat van GLRaV-3 geïnfekteerde wingerde is geïsoleer, gekloneer en die volgorde is bepaal. Dubbelstring RNA (dsRNA) was uit GLRaV-3 geïnfekteerde materiaal geëkstraheer en 'n hoë molekulêre gewig band van ~18 kb is geïdentifiseer. Die dsRNA is gebruik as 'n templaat vir 'n omgekeerde transkripsie PKR saam met GLRaV-3 CP geen spesifieke inleiers vir die amplifikasie van die GLRaV-3 CP geen (975 bp). Die GLRaV-3 CP geen is gekloneer in die pGem®-T Easy vektor. Klone met die CP geen in die sin (pLR3CP+) en teensin (pLR3CP-) oriëntasies respektiewelik is verkry. Die volgorde wat verkry is vanuit hierdie twee klone dui op 'n 99.26 % ooreenstemming met die enigste ander GLRaV-3 CP geen volgorde wat beskikbaar is. Die GLRaV-3 CP geen is uit pLR3CP+ en pLR3CP- gesny en is gesubkloneer in 'n plant ekspressie vektor, pCAMBIA 3301 in die sin (pCamBLR3CP+) en teensin (pCamBLR3CP-) oriëntasies respektiewelik, wat die sin en teensin geen ekspressie in transgeniese plante in staat stel. Die GLRaV-3 CP geen was ook gesubkloneer vanaf pCamBLR3CP+ in 'n ander plant ekspressie vektor, pCAMBIA 2301 in die sin orientasie en is as pCVSLR3CP+ benoem. Hierdie drie konstruksies is aan Dr. M. Vivier (Instituut vir Wyn Biotegnologie, Stellenbosch) gegee vir wingerd transformasie eksperimente. Twee van

hierdie konstruksies, pCamBLR3CP+ en pCamBLR3CP- asook pCAMBIA 3301 is gebruik om *Nicotiana tabacum* deur middel van *Agrobacterium tumefaciens*-bemiddelde transformasie te transformeer. Plante is geselekteer vir hul vermoë om die onkruiddoder, Basta, te weerstaan. Die teenwoordigheid van die plant selekteerbare merker geen, *bar*, op elke konstruksie lui tot dié weerstand. Die plante wat getransformeer is met pCamBLR3CP+ en pCamBLR3CP- is deur PKR saam met die GLRaV-3 CP geen spesifieke inleiers getoets, en geen amplifikasie van die GLRaV-3 CP geen is getoon nie. Southern blot analise met die GLRaV-3 CP geen as hibridisasie peiler het geen sein gewys vir hierdie plante nie, wat die PKR resultate bevestig. Die plante wat getransformeer is met pCAMBIA 3301 is deur PKR saam met die *bar* geen spesifieke inleiers getoets, en geen amplifikasie van die *bar* geen is getoon nie. Die plante wat getransformeer is met pCamBLR3CP+ en pCamBLR3CP- is ook getoets vir die teenwoordigheid vir die *bar* geen. Drie van die agt plante wat getoets is, het amplifikasie van die ~560 bp *bar* geen getoon. Hierdie onverwagte resultate stel voor dat dié plante met pCAMBIA 3301 (vektor sonder die geligeerde GLRaV-3 CP geen) en nie met pCamBLR3CP+ en pCamBLR3CP- getransformeer is nie. Hierdie projek verskaf voorlopige werk vir die daaropvolgende transformasie van wingerd met die GLRaV-3 CP geen in 'n poging om virus bestandheid te verskaf.

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ABBREVIATIONS

α	alpha
Amp	ampicillin
6-BAP	6-benzylaminopurine
bar	bialaphos
bp	base pairs
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CP	coat protein
CPd	divergent coat protein
CPMR	coat protein-mediated resistance
CTAB	N-Cetyl-N,N,N-trimethyl ammonium bromide
Cx	cefotaxime
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
g	gram
g	gravitational acceleration
GLR	grapevine leafroll
GUS	β-glucuronidase
HEL	helicase
HSP70/90h	heat shock protein 70/90 homologue
ICTV	International Committee on the Taxonomy of Viruses
kb	kilobases
KCl	potassium chloride
Km	kanamycin
lac Z	β-galactosidase Z
LB	Luria-Bertani

L-Pro	leader-proteinase
M	molar
MET	methyltransferase
$MgCl_2$	magnesium chloride
mg/l	milligram/litre
ml	millilitre
mM	millimolar
MP	movement protein
Mr	molecular weight
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog
1-NAA	1-napthaleneacetic acid
NaCl	sodium chloride
ng	nanogram
nm	nanometre
NOS	nopaline synthase
NPT II	neomycin phosphotransferase II
nt/s	nucleotide/s
OD	optical density
ORF/s	open reading frame/s
PAT	phosphinothricin acetyl transferase
PCR	polymerase chain reaction
PDR	pathogen-derived resistance
pg	picogram
POL	polymerase
PPT	phosphinothricin
PRO	proteinase
RE/s	restriction enzyme/s
Rif	rifampicin
RNase	ribonuclease
Rnasin	ribonuclease inhibitor
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
STE	sodium chloride, Tris, EDTA

TAE	Tris, acetic acid, EDTA
T-DNA	transfer deoxyribonucleic acid
Ti	tumour inducing
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloric acid
U	unit
µg	microgram
µg/ml	microgram/millilitre
µl	microlitre
µM	micromolar
V	volts
Vir	virulence

VIRUS NAMES AND ACRONYMS

ArMV	Arabis mosaic virus
ALMV	Alfalfa mosaic virus
BYSV	Beet yellow stunt virus
BYV	Beet yellows virus
CMV	Cucumber mosaic virus
CTV	Citrus tristeza virus
GAMaV	Grapevine asteroid mosaic-associated virus
GCMV	Grapevine chrome mosaic virus
GFkV	Grapevine fleck virus
GFLV	Grapevine fanleaf virus
GLRaV-1 to –8	Grapevine leafroll associated virus-1 to –8
GVA	Grapevine virus A
GVB	Grapevine virus B
GVC	Grapevine virus C
GVD	Grapevine virus D
GRGV	Grapevine redglobe virus
LChV	Little cherry virus
LIYV	Lettuce infectious yellows virus
PEBV	Pea early browning virus
PLRV	Potato leafroll virus
PRV	Papaya ringspot virus
PVX	Potato virus X
PVY	Potato virus Y
RSPaV	Rupestris stem-pitting associated virus
SMV	Soybean mosaic virus
TEV	Tobacco etch virus
TMV	Tobacco mosaic virus
TSWV	Tomato spotted wilt virus

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CHAPTER 1

INTRODUCTION

"NATURE IS TO BE FOUND IN HER ENTIRETY NOWHERE MORE THAN IN HER SMALLEST CREATURES"

PLINY
ROMAN WRITER

1.1 HISTORICAL BACKGROUND

Wild and cultivated grapevines belong to the family *Vitaceae*, which includes 11 genera and more than a thousand species. *Vitis* is the only genus being used in commercial viticulture and thus makes it economically the most important. *Vitis vinifera* L. is one of the most important species from this genus because of its fruit quality. It is thus the choice grapevine used for the production of wine.

During the period between the fourteenth and eighteenth centuries, the art of winemaking expanded throughout the world. Today the majority of grapes grown are used for the production of wine. The most recent statistics of the South African wine industry is for 1998 (http://www.wosa.co.za/Statistics.asp). South Africa is the sixth largest wine producing country in the world. During 1998, ~ 816 million litres of wine was produced, of which ~544 million litres was good wine for drinking purposes.

Unfortunately *Vitis vinifera* L. is susceptible to many pathogens, including viruses, that pose a major threat to the wine industry. The industry has identified grapevine leafroll (GLR) disease as the most devastating viral disease of grapevine in South Africa. If one looks at the following South African wine industry statistics: in 1998, the area under vines (wine grape varieties) was 100 979 hectares. In 1997 and 1996 these areas were 98 203 and 95 721 hectares respectively. Regardless of the increase in "wine vine" area from 1996 to 1998, the amount of grapes that were used for wine production decreased from 1 148 114 tons in 1996 to 1 120 602 tons in 1997 to 1 041 004 tons in 1998. Could this decrease in the amount of good quality wine grapes be attributed to viral diseases? The answer is off course debatable. Wine farmers, however, are becoming increasingly aware of, and concerned about the devastating effects of GLR and other viral diseases that are affecting their vineyards. The fact is that the damage caused by this disease is resulting in losses. These are difficult to determine, but are probably running into millions of rands annually in South Africa.

It has become the focus of many plant virologists to work towards developing resistance of grapevine to leafroll disease. We, like so many other scientists are aiming towards genetically modifying the crop to possibly obtain viral protection.

1.2 VIRUSES OF GRAPEVINE

Viruses are classified by the International Committee on the Taxonomy of viruses (ICTV) (http://life.anu.edu.au/viruses/ictv/index.html). All viruses get distinguished as either RNA or DNA viruses depending on whether their genomes consist of RNA or DNA. Furthermore, viruses are subdivided into groups and families depending on whether they possess a single or double strand of RNA or DNA of either positive or negative sense. The physicochemical, biological and serological properties as well as their mode of replication are used to further classify viruses into various genera.

To date, there are 47 viruses affecting grapevine, distributed in 17 plant virus genera, two of which are novel to *Vitis* and an eighteenth genus soon to be established (Martelli, 2000). A few of these will be mentioned briefly, the intention of which is to illustrate the most important and comprehensively studied viruses affecting grapevine worldwide.

Grapevine viruses A (GVA), B (GVB) and D (GVD), as well as the tentative species grapevine virus C (GVC) are classified in the genus *Vitivirus* (Martelli *et al.*, 1997), a derivative of the genus *Trichovirus* (Martelli *et al.*, 1994). This new genus was established as a result of molecular, biological and epidemiological differences between GVA, GVB (previously classified as a tentative species of the genus *Trichovirus*) and other definite species of the genus *Trichovirus* (Minafra *et al.*, 1997). The vitivirus filamentous virus particles are ~800 nanometres (nm) long and contain a single-stranded positive sense RNA molecule ~7600 nucleotides (nts) in size. GVA and GVB have the same genome size and structural organization. Both are phloem limited mechanically transmitted viruses and have pseudococcid mealybug vectors. GVA has been associated with Kober stem grooving (Chevalier *et*

al., 1995) and GVB with corky bark (Boscia *et al.*, 1993), two diseases of the grapevine rugose wood complex. Rugose wood has a high incidence in most viticultural regions of the world and is a complex disease of grapevine. The disease causes stunting, abnormal bark rugosity, delayed bud burst, dieback and abnormal swelling at the base of the scion in grafted vines (Habili & Symons, 2000). GVD is also associated with rugose wood, but many of the biological and epidemiological aspects are still poorly understood (Abou-Ghanem *et al.*, 1997).

The genus *Foveavirus* was established to accommodate viruses with a single-stranded RNA genome 8.4 kilobases (kb) to 9.3 kb in size and with filamentous particles ~800 nm long (Martelli & Jelkmann, 1998). Rupestris stem pitting associated virus (RSPaV) is the grapevine-infecting representative of this genus. The virus is strongly associated with, and is most probably the causal agent of rupestris stem pitting, a disease of the rugose wood complex (Meng *et al.*, 1999). The virus particles of RSPaV have apparently never been seen before (Martelli, 2000)! The rugose wood complex and especially RSPaV occurs worldwide with high incidences in the Mediterranean and Near East countries such as Italy (Digiaro *et al.*, 2000). RSPaV is also the most widespread virus affecting grapevines in Australia (Habili & Symons, 2000). The four viruses thus associated with the rugose wood complex of grapevine are GVA, GVB, GVD and RSPaV.

Grapevine fleck virus (GFkV), Grapevine asteroid mosaic associated virus (GAMaV), and the Grapevine Redglobe virus (GRGV) are positive sense single-stranded RNA, non-mechanically transmissible phloem limited viruses, serologically unrelated to one another. The virus particles are isometric and show typical surface structural characteristics of the genera *Tymovirus* and *Marafivirus*. However, because of phylogenetic differences based on nucleotide sequences between these three viruses and those of the *Tymovirus* and *Marafivirus* genera, a taxonomic allocation has not yet been assigned (Martelli, 2000). GFkV is widespread in Europe, especially Spain and it is the second most abundant virus found in Australian vineyards (Habili & Symons, 2000).

Viruses belonging to the genus *Nepovirus* are characterized by their isometric particles and by their transmission by soil-borne nematodes or, occasionally, seed. Their genome is divided between two single-stranded positive sense RNA molecules. The grapevine-infecting representatives of this genus are the grapevine fanleaf nepovirus (GFLV), Arabis mosaic virus (ArMV) and the Grapevine chrome mosaic nepovirus (GCMV) (Brault *et al.*, 1993). GFLV and ArMV together with other nepoviruses cause grapevine fanleaf disease known as "court-noué", one of the most widespread, economically damaging diseases of grapevine. Symptoms of the disease include malformed, asymmetrical, chlorotic leaves with abnormally gathered primary veins giving the leaf an appearance of an open fan. The disease results in poor fruit set and bunches that are smaller than usual, often with shot berries. It causes a progressive decline in the vineyards resulting in low yields (Goheen, 1988). Fortunately for the Australian grapevine farmers the vector needed to transmit GFLV is not present in Australia, but for most of the world especially France, grapevine fanleaf disease remains a serious problem (Vuittenez, 1985).

The genus *Closterovirus* represents filamentous plant viruses with very large RNA genomes of up to 20 kb. The grapevine-infecting representatives of this genus are the phloem limited grapevine leafroll associated closteroviruses-1 to -8 (GLRaV-1 to -8), which are strongly associated with GLR. As mentioned before, GLR has been identified as the most serious and devastating disease affecting grapevines in South Africa (Kriel, pers. comm.). The detail of this disease and the closteroviruses associated with it will be discussed in later sections.

The study of grapevine viruses remains a difficult task. Mixed viral infections in single vines are common and make the association of virus and symptom very complex. Also, different strains of the same virus are commonly found together in infected vines. Virus purification techniques are hampered by the high presence of phenolic compounds in grapevines. This, together with the phloem-bound nature of these viruses accounts for the limited availability of high quality antisera (Goszczynski *et al.*, 1996). Fortunately, scientists are not known to give up easily!

1.3 GRAPEVINE LEAFROLL DISEASE

Grapevine leafroll probably originated in the Near East and was carried west with grape cuttings of *Vitis vinifera*. In the mid-nineteenth century GLR was known in California as white Emperor disease and red leaf disease. These grapevine diseases reported to have affected the berry coloration, maturation and yield of the red table grape *Vitis vinifera* L. Emperor (Goheen *et al.*, 1958). A group of diseases known as *rollkrankheit* in Germany, *rougeau, flavescence, brunissure* and *enroulement* in France, showing these same symptoms, were also given the general name of leafroll disease (reviewed in Krake, 1993). GLR was suspected to be of viral origin, but the etiology remained undetermined for many years. In 1936 Scheu (reviewed in Ling *et al.*, 1997) showed that the disease was graft transmissible.

The symptoms of GLR are dependant on a number of factors, such as the virus isolate, environmental conditions and cultivar of grape. Characteristic phenotypic changes in symptomatic vines are interveinal reddening (red cultivars) and interveinal chlorosis (white cultivars) accompanied by a downward rolling of the leaf lamina (Fig. 1.1). GLR affects the quality of the grapes by delaying maturation and lowering the sugar content of the berries (Krake, 1993). Yield losses due to poor fruit set have varied in different regions but can be anything between 20-40 % in infected vines (Goheen, 1988, Woodham *et al.*, 1984). All *Vitis vinifera* varieties are susceptible to GLR and the symptoms are distinct in most European grape varieties (reviewed in Boscia *et al.*, 1995). American rootstocks are asymptomatic disease carriers, which result in American *Vitis* species and their hybrid cultivars being symptomless with the exception of one, namely *Vitis riparia* Gloire (Wilcox *et al.*, 1998).

Fig. 1.1. Photo of grapevine affected by grapevine leafroll disease. The interveinal reddening and downward rolling of the leaves can clearly be seen in the red grape cultivar.

As mentioned, the etiology of GLR was unknown for many years and in this time researchers from several countries associated various virus-like particles with GLR infected vines. These were: potyvirus-like particles (Tanne *et al.*, 1977), isometric virus-like particles (Castellano *et al.*, 1983) and closterovirus-like particles first observed by Namba *et al.*, 1979 (reviewed in Castellano *et al.*, 1983). In the years that followed, flexuous, filamentous closterovirus-like particles ranging from 1400-2200 nm long, were consistently identified in the phloem tissue of GLR affected vines (Castellano *et al.*, 1983, Conti & Milne, 1985, Gugerli *et al.*, 1984, Mossop *et al*, 1985, Hu *et al.*, 1990b, Milne *et al.*, 1984, Zee *et al.*, 1987, Zimmerman *et al.*, 1990). To date, there are eight serologically distinct closteroviruses, designated as GLRaV-1 to -8 (Boscia *et al.*, 1995, Choueiri *et al.*, 1996, Hu *et al.*, 1990a, Monis & Bestwick, 1997, Zimmerman *et al.*, 1990). Four of these are said to be genuine agents of GLR (GLRaV-1, -2, -3 & -7), of which GLRaV-3 is the most prevalent, whereas the other four are at this stage only associated with GLR (Grammatikaki &

Avgelis, 2000). There are two patents: the entire nucleotide sequence and proteins of GLRaV-3 (Ling & Gonsalves, 1999), US patent no. 05907085 (http://patent.womplex.ibm.com/details?pn=US05907085) and the 37 kilodalton (kDa) coat protein (CP) of GLRaV-8, as determined serologically (Monis & Bestwick, 1999) US patent no. 05965355 (http://patent.womplex.ibm.com/details?pn=US05965355).

It was originally thought that GLR was only transmitted by grafting healthy vines with infected rootstocks or scions (Goheen *et al.*, 1958), but unfortunately this is not the case. The first report of field spread of GLR was in 1973 by Dimitrijevic (reviewed in Engelbrecht & Kasdorf, 1990b) in Yugoslavia. Numerous reports since then have confirmed the field spread of GLR and the severity of this spread. In 1993, Jordan reported that GLR infections increased from 9 % to 93 % over a five-year period in a New Zealand vineyard. Certain areas on the globe show a much faster spread of GLR in the vineyards. In a vineyard in Northern Spain infections increased from 33 % to 83 % in just four years (Cabalero & Segura, 1997a & b), whereas in an Australian vineyard, infections increased from 23 % to 53 % in eleven years (Habili & Nutter, 1997). GLRaV-3 has predominantly been associated with GLR spread to healthy vines. In South Africa, particularly, this field spread of GLRaV-3 has reached epidemic proportions (Kriel, pers. comm.).

1.4 GENUS: *CLOSTEROVIRUS*

1.4.1 Taxonomy

The family *Closteroviridae* was established to accommodate positive sense RNA plant viruses with flexible filamentous particles and very large RNA genomes. The ICTV has subdivided this family into two genera: viruses with a monopartite genome, of which beet yellows virus (BYV) is the type member (genus *Closterovirus*) and viruses with a bipartite genome of which lettuce infectious yellows virus (LIYV) is the type member (genus *Crinivirus*). In the literature, LIYV is still referred to in many cases as a member of the genus *Closterovirus* (Klaassen *et al.*, 1995). All viruses classified in the genus

Closterovirus conforms to the typical structure of the type member, BYV and upon infection give rise to virus aggregates in the plant cells. The most characteristic of these inclusions is vesicles surrounded by a membrane known as BYV-vesicles. This is an important feature used in the taxonomic allocation of viruses in the family *Closteroviridae* (reviewed in Agranovsky, 1996, Dolja *et al.*, 1994 & http://life.anu.edu.au/viruses/ictv/index.html). Viruses are also divided into four supergroups based on similar features of viruses within that particular supergroup. These are, the alpha-like, picorna-like, carmo-like and sobemo-like groups (Goldbach *et al.*, 1991). The alpha-like supergroup includes the following plant virus genera: *Alfamovirus, Bromovirus, Capillovirus, Carlavirus, Closterovirus, Cucumovirus, Furovirus, Hordeivirus, Idaeovirus, Ilarvirus, Potexvirus, Tobamovirus, Tobravirus, Trichovirus and Tymovirus* (Zaccomer *et al.*, 1995).

The genus *Closterovirus* includes monopartite aphid-transmissible viruses with genomes of up to 20 kb. Some well known viruses from this group include the monopartite BYV which affects sugar beet crops (Agranovsky *et al.*, 1994) and citrus tristeza virus (CTV) (Karasev *et al.*, 1995) which causes major economic losses in the citrus fruit industry. The other group in this genus is the monopartite mealybug-transmissible viruses. Some well-known viruses in this group include the monopartite little cherry virus (LChV) (Jelkmann *et al.*, 1997) which affects cherries and GLRaV-3 (Ling *et al.*, 1997) which affects grapevine.

1.4.2 Particle morphology

Closteroviruses have very flexible filamentous particles ranging from 950-2000 nm long and ~12 nm in diameter (Fig. 1.2). The genome consists of a single molecule of RNA (monopartite) in a helical arrangement encapsidated by many identical CP subunits. They have the largest genomes among all RNA plant viruses, varying from ~7-20 kb in size (reviewed in Agranovsky, 1996). The CP subunits range in size from 22,3 kDa in BYV (Agranovsky *et al.*, 1991) to 46 kDa in LChV (Jelkmann *et al.*, 1997).

Fig. 1.2. Electronmicroscopy reveals ~1800-2000 nm long particles of the grapevine leafroll associated closterovirus-3. The flexuous, filamentous virus particles are typical of the family *Closteroviridae* (Photograph courtesy of G. Kasdorf).

1.4.3 Replication

The replication strategy of a positive sense RNA virus is described here in a very simplified manner. When the virus infects a plant cell it is released from the protein coat and induces the plant cells' machinery to form viral RNA polymerase, the product of the RNA dependent RNA polymerase gene situated in the viral genome. This enzyme uses the viral positive RNA strand as a template for the formation of a complementary RNA strand (minus strand). This strand is a mirror image of the viral positive RNA strand and is temporarily bound to it to form double stranded RNA (dsRNA). These two strands split soon after formation and the minus RNA strand serves as a template for the formation of a new positive viral RNA strand. The new viral nucleic acid serves as a messenger RNA (mRNA) and utilizes the host plant cells ribosomes, amino acids and transfer RNA molecules to induce expression of proteins from the viral RNA genome. The new viral nucleic acid

arranges the new structural CP subunits around it, and are assembled together to form a new complete virus particle, the virion. The replication and viral assembly of positive strand RNA viruses takes place in the cytoplasm of the host plant cell (Agrios, 1988).

1.4.4 Genome organization and gene expression

Sequence analysis of the genomes of some mono- and bipartite viruses in the family *Closteroviridae* have shown that the CP gene is duplicated, known as the divergent coat protein (CPd). It has become a unique feature of this group of viruses (Klaassen *et al.*, 1995, Keim-Konrad & Jelkmann, 1996, Zhu *et al.*, 1998, Ling *et al.*, 1998, Agranovsky *et al.*, 1994, Karasev *et al.*, 1995, reviewed in Agranovsky, 1996 & Dolja *et al.*, 1994). This deviates from the consensus that a single viral gene encodes the CP subunits. Studies on BYV (Agranovsky *et al.,* 1994) and CTV (Febres *et al.,* 1996) shows that the CPd gene encodes minor CP subunits, which are arranged at one end of the virus particle resulting in a protein tail giving the appearance of a "rattlesnake". The function of the CPd is not clear but it has been proposed to be involved with long distance and cell-to-cell movement of a viral infection rather than in the formation of the coat protein (reviewed in Dolja *et al.*, 1994). Recently Zinovkin *et al.* (1999) proposed that after transmission of the virus to the host plant cell, this "rattlesnake" protein tail dissociates from the virus particle exposing only a small piece of the RNA genome, making it accessible to the ribosomes for translation. At this time, the rest of the virus particle remains covered with major CP subunits encoded by the CP gene, but will slowly be stripped by the translating ribosomes as proposed by the co-translational disassembly model of the tobacco mosaic virus (TMV) (Wilson, 1984). It has been suggested that this mechanism of stepwise unwrapping of the closterovirus particles protect the virus RNA genome from cell nucleases.

Features characteristic of a closterovirus genome will be mentioned, but since the BYV is the prototype closterovirus, a brief description of the genome organization is mentioned (Fig. 1.3). Most of the information regarding the functions of the proteins expressed in a closterovirus genome have been

inferred by computer assisted analysis and studies on BYV (Peremyslov *et al.*, 1998).

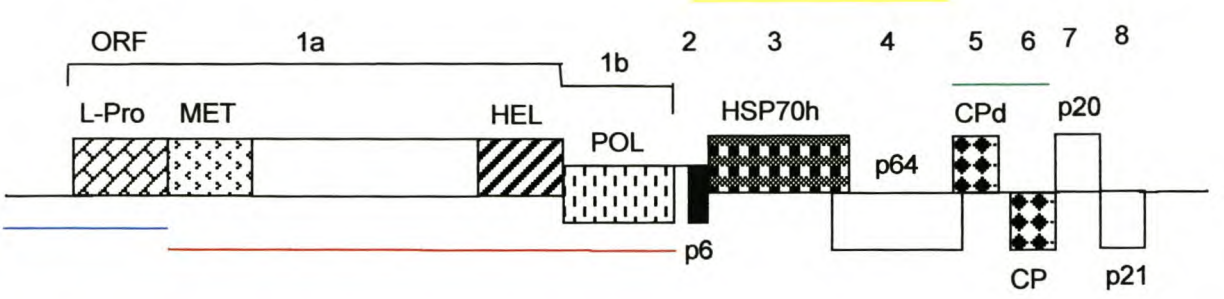

Fig. 1.3. Genome organization of BYV. The genome of BYV is divided into 9 open reading frames (ORFs) (1a and b, 2-8). L-Pro, leader papain-like proteinase; MET, methyltransferase; HEL, helicase; POL, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homologue; CPd, divergent coat protein; CP, coat protein.

The closterovirus genome is tentatively divided into four modules as proposed by Dolja *et al.* (1994). The core module (red line in Fig. 1.3) consists of three domains, which collectively make up the replicase complex: the methyltransferase (MET), helicase (HEL) and RNA-dependent RNA polymerase (POL). The core module is conserved throughout the alphavirus-like supergroup. The accessory module (blue line in Fig. 1.3) encoding a papain-like protease and a putative vector-specificity factor is situated on the 5' end upstream of the core module. The molecular chaperone module (yellow line in Fig. 1.3) encodes for the cellular heat shock protein 70 homologue (HSP70h) and a distantly related heat shock protein 90 homologue (HSP90h). This chaperone-CP module is conserved throughout the closteroviruses. The fourth module (green line in Fig. 1.3) is a structural module that contains the CP gene and its duplicate. All viruses have a CP gene but as mentioned before, the CPd gene is unique to closteroviruses. These modules have been widely regarded as consistent among the closteroviruses, however Agranovsky (1996) suggested the genome division

comprise three modules, the difference being the fusion of the molecular chaperone and structural modules.

L-Pro gene, ORF 1a in BYV: The L-Pro gene encodes a proteinase, which is used for the post-translational autoproteolytic cleavage of the other functional protein domains. This is used in RNA viruses as a strategy for expression. It was shown by site directed mutagenesis that the cleavage site region was imperative for virus replication and that the central domain of L-Pro has various accessory functions during virus reproduction (Peremyslov *et al.*, 1998). The L-Pro domain in BYV encodes a papain-like proteinase, which is distantly related to the helper component-proteinases of potyviruses. The N-terminal domain of the helper component-proteinase appears to be involved in transmission of potyviruses by their aphid vectors. The L-Pro in closteroviruses may have similar functions (reviewed in Dolja *et al.*, 1994). Approximately one fourth of the C-terminal region of the L-Pro domain is conserved among the closteroviruses (Karasev *et al.*, 1995, Agranovsky *et al.*, 1994).

MET, HEL and POL domains, ORF 1a and b in BYV: Site directed mutagenesis experiments on a full-length complementary DNA (cDNA) clone of BYV showed that expression of the MET, HEL and POL domains were essential for genome amplification during virus replication (Peremyslov *et al.*, 1998). Features characteristic of the closterovirus genome are the large intergenic region between the MET and HEL domains in ORF 1a and the involvement of a +1 translational ribosomal frameshift in the expression of the ORF 1a/1b fusion protein (Peremyslov *et al.*, 1998). This frameshift has been observed in BYV (Agranovsky *et al.*, 1994), CTV (Karasev *et al.*, 1995), LIYV (Klaassen *et al.*, 1995), beet yellow stunt virus (BYSV) (Karasev *et al.*, 1996), LChV (Jelkmann *et al.*, 1997) and GLRaV-3 (Ling *et al.*, 2000).

ORF 2 in BYV: This gene encodes a small hydrophobic transmembrane protein (Agranovsky *et al.*, 1991). A small hydrophobic protein has also been observed in CTV and LIYV (Karasev *et al.*, 1995).

HSP70h gene, ORF 3 in BYV: Closteroviruses are the only viruses that encode homologues of the HSP70 and HSP90 families of heat shock proteins (Tian *et al.*, 1996). The family of HSP70 genes encodes ubiquitous molecular chaperone-like or movement proteins (MP), which are present in all cells. Each protein consists of functionally important motifs. The N-terminal domain of the protein possesses ATPase activity and the C-terminal domain is responsible for protein-protein interactions (Agranovsky *et al.*, 1997, Bork *et al.*, 1992). There are eight conserved motifs in the HSP70h proteins among the closteroviruses. The functions of the HSP70h and HSP90h of closteroviruses still remain unclear, but it has been suggested that these protein homologues participate in cell-to-cell movement of viruses (Agranovsky *et al.*, 1998, Peremyslov *et al.*, 1999).

CP, CPd genes, ORF 5 & 6 in BYV: The functions of the CP and CPd genes have been discussed (refer to par. 1.4.4.). There are four conserved amino acids (N, R, G & D) found in all closterovirus CPs (Dolja *et al.*, 1991). They are crucial for protein structure and may be involved in salt bridge formation. It has also been shown that they are involved in maintaining the conserved hydrophobic core region of the CPs of all filamentous plant viruses.

Other genes in ORF 2-8 in BYV: The genes in these ORFs are expressed via a nested set of subgenomic RNAs, which has also been observed in CTV (reviewed in Dolja *et al.*, 1994). Site directed mutagenesis experiments on a full-length cDNA clone of BYV showed that the other genes in ORFs 2-8 were not required for amplification of viral RNA during replication. Lower amounts of accumulated RNA were however observed. The protein designated as p21 has been identified to enhance the accumulation of RNA during viral replication. The protein designated as p6 is a hydrophobic protein (Peremyslov *et al.*, 1998).

1.5 GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUSES-1 TO -8

A comparative analysis regarding important research aspects of the eight GLRaVs is given in table format (Table 1.5.1, 1.5.2, 1.5.3 & 1.5.4). To date,

the GLRaVs have been distinguished based on their serological and not their molecular characteristics. As a general consensus, all eight closteroviruses are serologically distinct, however, it has been reported that monoclonal antibodies to GLRaV-1 reacted to GLRaV-3. This is the first report of a distant serological relationship between any of the GLRaVs (reviewed in Martelli, 2000).

1.6 GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS-3

As has been mentioned, GLRaV-3 is the best characterized and most important of the grapevine closteroviruses. It is the most strongly associated with leafroll spread in a vineyard. One could thus unofficially refer to GLRaV-3 as the "type member" of the grapevine closteroviruses. This section is therefore dedicated to the aspects of GLRaV-3 that have not been discussed in detail, such as the genome organization. It is also the virus that has been targeted for this project.

1.6.1 Physicochemical characteristics

The virus particles of GLRaV-3 are about 1800-2000 nm long. The molecular weight (Mr) of the CP subunits as determined electrophoretically is ~43 kDa (Ling *et al.*, 1997) and as determined by sequence analysis is ~35 kDa (Martelli, 2000).

1.6.2 Genome organization

Sequence analysis of GLRaV-3 has revealed that there are 13 ORFs designated as 1a, 1b and 2-12 (Fig. 1.4). The genome of GLRaV-3 consists of a high Mr dsRNA of ~18 kb, which is consistent of a typical monopartite closterovirus. Of the total 17 919 nts, there is a 5' untranslated region of 158 nts and a 3' untranslated region of 276 nts. The entire sequence of GLRaV-3 has been deposited in Genbank: accession number AF037268 (Ling *et al.*, 1998, Ling *et al.*, 2000).

Table 1.5.1. Comparative analysis of the GLRaVs: GLRaV-1 & GLRaV-2

	Grapevine leafroll associated virus-1	Grapevine leafroll associated virus-2
Etiology	Closterovirus considered as a genuine agent of GLR. One of the most widespread and economically important of the GLRaVs. The word "associated" may soon be dropped from the name (Boscia *et al.*, 1995).	Closterovirus considered as a genuine agent of GLR (Grammatikaki & Avgelis, 2000). It is also associated with graft incompatibility disorders in vines. Three biological strains of GLRaV-2 exist (Goszczynski *et al*, 1996, Abou-Ghanem *et al.*, 2000). Differs from the other GLRaVs because of the outward appearance of the particles and the low Mr of the CP subunits (Boscia *et al.*, 1995).
Molecular biology	The genome of GLRaV-1 has been cloned and 12 394 nts sequenced (Fazeli & Rezaian, 2000). Ten ORFs have been identified, which matches the organization of BYV. Three of the four genome modules have been identified. These consist of a part of the replication machinery (HEL and POL domains), the HSP70 and HSP90 homologues, the CP gene and 2 copies of the CPd gene. The CPd genes occur in 2 ORFs and this dual duplication has not been reported for the other GLRaVs. The Mr of the CP is ~38 kDa. Phylogenetic analysis of the CP gene nt sequence shows a close relationship to GLRaV-3. There is a large degree of diversity in the nt sequence between different isolates of GLRaV-1, specifically ORFs 3, 6 and 7 (Little *et al.*, 2000). These differences result in amino	The complete genome has been cloned and sequenced (Abou-Ghanem *et al.*, 1998, Gonsalves, 2000, Meng *et al.*, 2000, Zhu *et al.*, 1998). The genome has 15 528 nts with 9 ORFs and matches the genome organization of BYV. The four genome modules in GLRaV-2 consist of a papain-like protease domain at the 5' end, the replication machinery (MET, HEL and POL domains), the HSP70 and HSP90 homologues and the CP and CPd genes (Gonsalves, 2000). The Mr of the CP deduced from sequence analysis is 22 kDa.

Table 1.5.1 continued

	GLRaV-1 continued	GLRaV-2 continued
Molecular biology continued	acid changes, but do not cause a frameshift in the ORFs. The replication functions of the translation products are therefore maintained.	
Mechanical and graft transmission	GLRaV-1 is graft transmissible. GLRaV-1 can not be mechanically transmitted to herbaceous plant species (Boscia *et al.*, 1995).	GLRaV-2 is graft transmissible. It is the only GLRaV that can be mechanically transmitted to herbaceous plant species e.g. *N. benthamiana* (Goszczynski *et al.*, 1996) and *N. occidentalis* (Abou-Ghanem *et al.*, 2000).
Vector transmission	It is transmitted by the scale insects *Neopulvinaria innumerabilis* and *Parthenolecanium corni* (Fazeli & Rezaian, 2000, Sforza *et al.*, 2000). It has recently been reported in France that the mealybugs, *Heliococcus bohemicus* Sulc and *Phenacoccus aceris* Signoret also transmit GLRaV-1 (Sforza *et al.*, 2000). None of the mealybug species mentioned for GLRaV-3 have been able to transmit GLRaV-1 (Golino *et al.*, 2000).	None of the mealybug species mentioned for GLRaV-3 have been able to transmit GLRaV-2 (Golino *et al.*, 2000).
Transformation experiments	No literature available.	*N. Benthamiana* and grapevine have been transformed with the CP gene of GLRaV-2. Transgenic tobacco plants were resistant to the virus. Transgenic grapevines are still in the screening phase (Gonsalves, 2000).

Table 1.5.2. Comparative analysis of the GLRaVs: GLRaV-3 & GLRaV-4

	Grapevine leafroll associated virus-3	Grapevine leafroll associated virus-4
Etiology	Closterovirus considered to be a genuine agent of GLR (Grammatikaki & Avgelis, 2000). The most widespread and economically important of the GLRaVs. The word "associated" may soon be dropped from the name (Boscia *et al.*, 1995).	Closterovirus associated with GLR in affected vines.
Molecular biology	The complete genome consisting of 17 919 nts has been sequenced (Ling *et al.*, 1998, Gonsalves & Ling, 1999, Ling *et al.*, 2000). There are 13 ORFs matching the genome organization of BYV. The four modules in the GLRaV-3 genome consist of a proteinase at the 5' end, the replication machinery (MET, HEL and POL domains), the HSP70 and HSP90 homologues and the CP and CPd genes. The PRO domain of GLRaV-3 does not encode a papain-like proteinase, but instead has a proteinase similar to that of the hepatitis C virus. The 3' portion of GLRaV-3 genome is expressed via a set of subgenomic mRNAs (Gonsalves, 2000). The Mr of the CP deduced from sequence analysis is ~35 kDa.	Degenerate primers have been produced to amplify a portion of the HSP70h gene. The GLRaV-4 cDNA clones derived from amplification have provided little sequence data, but proves that GLRaV-4 is indeed a closterovirus (Routh *et al.*, 1998). The Mr of the CP, as determined serologically is ~36 kDa (Monis & Bestwick, 1999).
Mechanical and graft transmission	GLRaV-3 is graft transmissible. It can not be mechanically transmitted to herbaceous plant species (Boscia *et al.*, 1995).	GLRaV-4 is graft transmissible. It can not be mechanically transmitted to herbaceous plant species (Boscia *et al.*, 1995).

Table 1.5.2 continued

	GLRaV-3 continued	GLRaV-4 continued
Vector transmission	GLRaV-3 is transmitted by mealybugs: *Planococcus citri* in Spain (Cabalero & Segura, 1997a & b), *Pseudococcus calceolariae* and *Pseudococcus longispinus* in New Zealand (Petersen & Charles, 1997), *Planococcus ficus* Signoret in South Africa and Sicily (Engelbrecht & Kasdorf, 1990a), *Pseudococcus maritimus*, *Planococcus citri*, *Pseudococcus viburni*, *Pseudococcus affinis* and *Pseudococcus longispinus* in California (Golino *et al.*, 1998, Golino *et al.*, 2000). The scale insect, *Pulvinaria vitis* L. is reported to also transmit GLRaV-3 in Italy (reviewed in Habili & Nutter, 1997).	None of the mealybug species mentioned for GLRaV-3 have been able to transmit GLRaV-4 (Golino *et al.*, 2000).
Transformation experiments	Tobacco has been transformed with the CP gene of GLRaV-3 to test for expression. GLRaV-3 can not be transmitted to herbaceous plants therefore resistance can not be evaluated in tobacco. Grapevine has also been transformed with the CP gene of GLRaV-3, but the plants are still in the screening phase (Gonsalves, 2000, Vivier, pers. comm.).	No literature available.

Table 1.5.3. Comparative analysis of the GLRaVs: GLRaV-5 & GLRaV-6

	Grapevine leafroll associated virus-5	Grapevine leafroll associated virus-6
Etiology	Closterovirus associated with GLR in affected vines.	Closterovirus associated with GLR in affected vines, although generally it has a low incidence. Reports thus far show an infection rate not exceeding ~2.6 % in all wine grape varieties (with the exception of one) tested. The cultivar *Vitis vinifera* Cardinal, however, shows a very high infection rate and has a very strong relationship with GLRaV-6 (Boscia *et al.*, 2000).
Molecular biology	Degenerate primers have been produced to amplify a portion of the HSP70h gene. The GLRaV-5 cDNA clones derived from amplification have also provided little sequence data, but proves that GLRaV-5 is indeed a closterovirus (Routh *et al.*, 1998). The Mr of the CP, as determined serologically, is ~36 kDa (Monis & Bestwick, 1999).	Due to the strong presence of other GLRaVs in vines infected with GLRaV-6, progress in the molecular characterization is slow (Boscia *et al.*, 2000). The Mr of the CP is ~32 kDa (reviewed in Martelli, 2000).
Mechanical and graft transmission	GLRaV-5 is graft transmissible. It can not be mechanically transmitted to herbaceous plant species (Boscia *et al.*, 1995).	GLRaV-6 is graft transmissible. It can not be mechanically transmitted to herbaceous plant species (Boscia *et al.*, 1995).
Vector transmission	No literature available.	No literature available.
Transformation experiments	No literature available.	No literature available.

Table 1.5.4. Comparative analysis of the GLRaVs: GLRaV-7 & GLRaV-8

	Grapevine leafroll associated virus-7	Grapevine leafroll associated virus-8
Etiology	Closterovirus considered to be a genuine agent of GLR (Grammatikaki & Avgelis, 2000).	Closterovirus associated with GLR in affected vines.
Molecular biology	GLRaV-7 has been partially sequenced and a few ORFs identified (Turturo *et al.*, 2000). The putative translation products of these ORFs, reveal similarities to those of GLRaV-2, GLRaV-3 and BYV. In particular, homologies to the MET and HEL domains and the CP genes of the above mentioned viruses were found. Further sequencing needs to be performed before the genome organization can be elucidated.	A 37 kDa protein from GLR infected vines was found to be immunologically distinct from the ~36 kDa proteins associated with GLRaV-4 & -5 and to the ~38 kDa CP of GLRaV-1. It has been confirmed that this is the CP of GLRaV-8 (Monis & Bestwick, 1997 & 1999). There is no sequence data available yet.
Mechanical and graft transmission	GLRaV-7 is graft transmissible. It can not be mechanically transmitted to herbaceous plant species (Boscia *et al.*, 1995).	No literature available.
Vector transmission	No literature available.	No literature available.
Transformation experiments	No literature available.	No literature available.

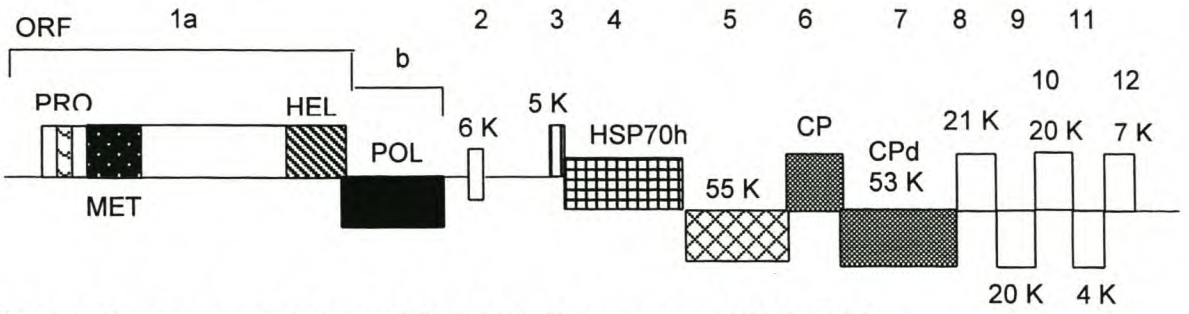

Fig. 1.4. Genome organization of GLRaV-3. The genome of GLRaV-3 is divided into 13 open reading frames (ORFs) (1a and b, 2-12). PRO, proteinase; MET, methyltransferase; HEL, helicase; POL, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homologue; CP, coat protein; CPd, divergent coat protein.

ORF 1a and 1b: ORF 1a consists of 6714 nts and encodes a large polyprotein with a Mr of 245,277 kDa. There exists a +1 frameshift and it is thus also possible to produce a very large fusion protein, which encompasses ORF 1a and 1b with a Mr of 305,955 kDa (Ling *et al.*, 2000). The so-called slippery sequence, GGGUUU, and the stem loop structure suggested to be involved in the frameshift in BYV is absent in GLRaV-3. There is a significant amino acid sequence similarity between the frameshift region of GLRaV-3 and LIYV but not to BYV or CTV (Klaassen *et al.*, 1995). This similarity possibly indicates that the frameshift in GLRaV-3 occurs in the homologous region of the LIYV genome (Gonsalves & Ling, 1999). ORF 1b overlaps the last 113 nts of ORF 1a. The PRO domain (ORF 1a) of GLRaV-3 does not encode a papain-like proteinase, but instead has a proteinase similar to that of the hepatitis C virus. The vector specificity factor is yet to be identified in this region (Ling *et al.*, 2000). The HEL (ORF 1b) has a Mr of ~148,5 kDa. Phylogenetic analysis of the HEL domains between GLRaV-3 and the other positive strand RNA viruses places GLRaV-3 together with the other closteroviruses into the "tobamo" branch of the alpha-like supergroup. The six conserved motifs present in the HEL domain of positive strand RNA plant viruses were shown to also exist in GLRaV-3 (Gonsalves & Ling, 1999). ORF 1b encodes the POL and has a Mr of 61,050 kDa. Phylogenetic analysis of the POL domains between GLRaV-3 and the other positive strand RNA viruses places GLRaV-3 together with the other closteroviruses again into the "tobamo" branch of the

alpha-like supergroup. Amino acid sequence comparison of the POL domains between GLRaV-3 and BYV, LIYV, LChV, BYSV and CTV shows that there is a similarity among eight conserved motifs (Ling *et al.*, 1998).

ORF 2: This ORF encodes a small peptide with a Mr of 5,927 kDa (Ling *et al.*, 1998). A long intergenic region of 1067 nts follows this ORF. There exists no counterpart ORFs in the genomes of BYV or LChV, but there is larger ORFs present in the same position in the genomes of CTV (Karasev *et al.*, 1995), LIYV (Klaassen *et al.*, 1995) and BYSV (Karasev *et al.*, 1996).

ORF 3: This ORF encodes a small peptide of 45 amino acids with a Mr of 5,090 kDa. This peptide is closely related to the small hydrophobic transmembrane proteins of BYV, CTV and LIYV (Karasev *et al.*, 1995). At the nucleotide level it is related the closest to LIYV and at the amino acid level it is the most homologous to BYV (Ling *et al.*, 1998, Gonsalves & Ling, 1999).

ORF 4: This ORF encodes a protein of 549 amino acids with a Mr of 59,113 kDa. There is a significant similarity between this protein and the HSP70 family of molecular chaperone proteins as well as to the HSP70 homologues of BYV, CTV and LIYV. There is also a striking amino acid sequence similarity between the eight conserved motifs of GLRaV-3 and these closteroviruses. Functionally important motifs characteristic of all the HSP70 chaperone proteins possessing an ATPase domain were also present in GLRaV-3. This suggests that the HSP70h in GLRaV-3 also has ATPase activity and that it may also be involved in protein-protein interactions (Ling *et al.*, 1998, Gonsalves & Ling, 1999).

ORF 5: This ORF encodes a protein of 483 amino acids with a Mr of 54,852 kDa. There is very little amino acid sequence similarity between this protein and the HSP90 homologues of BYV, CTV and LIYV. There is also no significant sequence similarity to any other proteins in the GenBank database. The two conserved regions between the HSP90 homologues of BYV and CTV are not present in this protein (Ling *et al.*, 1998). (Due to a contradiction in the GLRaV-3 patent (Gonsalves & Ling, 1999), stating that the two conserved

regions were indeed present in this GLRaV-3 protein, we did an amino acid sequence alignment of this protein with the HSP90 homologues of BYV and CTV. The publication by Ling *et al.* (1998) is indeed correct; the two conserved regions are not present in this GLRaV-3 protein).

ORF 6: This ORF consists of the CP gene, which encodes the CP subunits. The protein consists of 313 amino acids and has a Mr of 34,866 kDa. The four conserved amino acids (N, R, G & D) are present in the amino acid sequence of the CP of GLRaV-3. Besides these, there is little sequence similarity between the CP of GLRaV-3 and those of LIYV, LChV, CTV, BYV and BYSV (Ling *et al.*, 1998). Phylogenetic analysis with regard to the CP gene sequence, places GLRaV-3 into a separate but closely related branch of the closteroviruses.

ORF 7: This ORF encodes a protein of 477 amino acids with a Mr of 53,104 kDa. It has been identified as the CPd gene (p59). The predicted functions have been discussed in a previous section. The four conserved amino acids (N, R, G & D) present in the C-terminal portion of all CPds of closteroviruses are also present in the amino acid sequence of the CPd of GLRaV-3 (Ling *et al.*, 1998). Phylogenetic analysis with regard to the CPd gene sequence, places GLRaV-3 again into a separate but closely related branch of the closteroviruses.

ORF 8: This ORF encodes an undefined protein of 185 amino acids with a Mr of 21,248 kDa (Ling *et al.*, 1998).

ORF 9: This ORF encodes an undefined protein of 177 amino acids with a Mr of 19,588 kDa (Ling *et al.*, 1998).

ORF 10: This ORF encodes an undefined protein of 179 amino acids with a Mr of 19,652 kDa (Ling *et al.*, 1998).

ORF 11: This ORF encodes an undefined protein of 36 amino acids with a Mr of 3,933 kDa (Ling *et al.*, 1998).

ORF 12: This ORF encodes an undefined protein of 60 amino acids with a Mr of 6,768 kDa (Ling *et al.*, 1998).

Two important factors prove that GLRaV-3 is indeed a closterovirus of the alphavirus-like supergroup. First, the core module (MET-HEL-POL) is conserved in the genome of GLRaV-3, which places GLRaV-3 in the alphavirus-like supergroup. Second, the chaperone-CP module (HSP70h-HSP90h-CP-CPd) is also conserved in the genome of GLRaV-3, which places GLRaV-3 in the closterovirus genus (Ling *et al.*, 1998).

1.7 STRATEGIES OF VIRUS CONTROL

1.7.1 Sanitation

Virus control measures, which exist today in the grapevine industry are to a large extent inefficient and often the virus wins the battle. Viruses can not be eradicated by the use of chemicals. The application of pesticides is therefore limited to the insect vectors that serve as host to the virus. However, the uncertainty about many of these vectors, for example, those of the GLRaVs, remains a problem in using this approach. The introduction of natural resistance in grapevine by conventional breeding seems such an unattainable prospect, that no such breeding program exists in South Africa, nor, to our knowledge, anywhere else in the world.

The only approach with a limited efficiency implemented in viticultural regions around the world, are sanitation programs that ensure the production of certified grape propagation material. In fact, member countries of the European Union, by law require all grapevine reproduction material to be free of "noxious" viruses before any trade is admitted. This places profound pressure on nurseries to supply high quality material to a market that has developed high expectations due to advancements in technology. Various protocols such as the polymerase chain reaction (PCR), enzyme linked immunosorbent assays (ELISA) and biological indexing on woody indicators are been used for the detection of viruses and virus-like diseases, but these

are purely preventative measures (Rumbos *et al.*, 2000). As mentioned earlier, when new vineyards are established in a leafroll-prevalent area using virus free propagation material, re-infection at a rapid pace is very likely to occur. There have been successes in eliminating viruses such as GFLV, GLRaV-1, GLRaV-3, GVA, GVB and GFkV from grapevine using *in vitro* shoot tip culture and heat treatment (Bottalico *et al.*, 2000, Buciumeanu & Visoiu, 2000). These two procedures are the most effective when used in combination with each other.

1.7.2 Genetic modification

1.7.2.1 Naturally occurring resistance genes

Genetic modification of crop species by selection and breeding has been used successfully in the past to provide differing degrees of resistance to viral, fungal and bacterial diseases. There have been several naturally occurring resistance genes isolated in the past. An example of one such gene, which has been used successfully to avoid the effects of a viral disease, is the tobacco *N* gene, which confers resistance to TMV (Whitham *et al.*, 1994). In spite of successes, there exist problems with this approach. First, there may not be a gene conferring resistance to a particular virus present in the plant and second, if there is, there has to be a corresponding avirulence gene present in the pathogen concerned (Jones, 1996).

1.7.2.2 Cross protection

There is another naturally occurring phenomenon of protection against viral diseases, known as cross protection. This occurs when the presence of a mild strain of a virus in a plant protects the plant from developing symptoms when it becomes infected by a more virulent strain of the same virus. This concept has been used successfully in the past to control viral diseases in a number of crop species, for example, resistance to CTV in citrus fruit trees (Costa & Müller, 1980). Cross protection has been shown to be effective against closely related strains, but not against distantly related viruses or virus strains (Wen *et al.*, 1991). No success with cross protection to GLRaV-3 has

been achieved in South Africa, nor, to our knowledge, anywhere else in the world (Carstens, pers. comm.).

1.7.2.3 Pathogen-derived resistance

In 1985, Sanford and Johnston developed the concept of pathogen-derived resistance (PDR) (Sanford & Johnston, 1985). As the name implies, resistance to a disease in transgenic plants is as a result of the expression of nucleotide sequences derived from the pathogen. PDR can be divided into two resistance strategies: those that require the expression of a protein and those that require only the accumulation of viral nucleic acids. The CP gene of TMV was used in the first demonstration of PDR in transgenic tobacco plants (Powell-Abel *et al.*, 1986). This form of PDR is known as coat protein-mediated resistance (CPMR) and requires the expression of a protein (reviewed in Hackland *et al.*, 1994). It is the most frequently used strategy and has been conferred to viruses in at least 13 RNA virus genera, including 23 distinct virus species (Grumet, 1995). Three CPMR transgenic crops have recently been approved for commercial release in the United States of America (reviewed in Aaziz & Tepfer, 1999). The production of genetically modified crops is, although much debated, an integral part of overcoming serious pathogenic problems. PDR is especially useful when no naturally occurring resistance genes have been identified, such as in grapevine (Gölles *et al.*, 2000).

The cellular and molecular mechanisms involved in PDR including CPMR still remain unclear. It appears however, that the resistance is due to multiple mechanisms operating and that it involves the early stages of viral infection (Register & Beachy, 1988). CPMR to TMV has become the model for hypotheses on the mechanisms of CPMR. An accumulation of CP in the transgenic tobacco plants is indispensable to provide resistance to TMV (Powell *et al.*, 1990). When the transgenic plants were inoculated with TMV RNA, the protection was largely but not completely overcome. The TMV RNA overcame the resistance to a large degree because the RNA is not encapsidated and therefore does not have to be disassembled for systemic infection to occur (refer to par. 1.4.3). Some cases of CPMR are able to

protect the transgenic plant against both the virion inoculum and RNA. An example of this is CPMR to potato virus X (PVX) (Hemenway *et al.*, 1988).

Data obtained from CP expression and accumulation in transgenic tobacco plants and protoplasts support the "inhibited-uncoating" hypothesis. This hypothesis suggests that the challenge virus is inhibited from uncoating and is consequently unable to expose the RNA genome to the plants cellular replication machinery (Osbourn *et al.*, 1989, Register & Beachy, 1988, Wu *et al.*, 1990). Two theories exist to explain the hypothesis. The first one suggests that the endogenous CPs assemble to form virus-like particles, which blocks an "uncoating receptor" on the challenge virus, thus inhibiting uncoating. It has been shown that a mutant form of the TMV CP, which lacks the capacity to assemble virus-like particles, either does not confer CPMR at all in transgenic tobacco plants, or if it does, the resistance is very low (Bendahmane *et al.,* 1997). The second theory suggests that uncoating is the result of an equilibrium shift, making it reversible. Therefore, when the challenge virus undergoes uncoating in the transgenic plant, the high concentration of endogenous CP in the plant re-encapsidates the challenge virus RNA, constantly shifting the disassembly-assembly reaction in favour of assembly, thereby preventing virus infection (Register & Nelson, 1992, Clark *et al.*, 1995a & b). This theory suggests that an important component of CPMR is a result of interactions between the endogenous CP subunits in the transgenic plant and the CP subunits of the infecting virus. Supporting this, studies demonstrated that the stronger these interactions and the CP accumulation, the greater the resistance of the transgenic plant (Bendahmane *et al.*, 1997). It has been reported in many cases that the lower the level of expression of the transgenic CP, the lower is the level of resistance. This has been seen, for example, in transgenic potato plants resistant to PVX (Hemenway *et al.*, 1988).

CPMR can provide either "broad" or "narrow" protection. It has been reported that CPMR mediated by the TMV CP is more effective against closely related strains of TMV or tobamoviruses with CP sequence similarity to TMV, than to those with a CP sequence dissimilar to the TMV CP sequence (Nejidat &

Beachy, 1990). Another example is the CP gene of papaya ringspot virus (PRV), which provides resistance only to that particular strain (Tennant *et al.*, 1994). These cases of CPMR provide "narrow" protection. In contrast though, expression of a single virus CP gene may provide differing degrees of resistance to other viruses, for example, the CP gene of soybean mosaic virus (SMV) confers resistance in tobacco to two totally unrelated potyviruses, potato virus Y (PVY) and tobacco etch virus (TEV) (Stark & Beachy, 1989). This case of CPMR provides a "broad" protection.

Another manifestation of CPMR is the delay of virus spread from inoculated leaves to the upper leaves of the plant. In a systemic viral infection, viruses move short distances from cell-to-cell through plasmodesmata in the cell walls with the aid of movement proteins and long distances through the vascular system (Hull, 1989). Saito *et al.* (1990) showed that the TMV CP and the assembly origin in the TMV RNA genome were responsible for the long distance movement of the virus. A mutation at certain positions in the TMV CP or the assembly origin in the TMV RNA caused a delay in the systemic spread of the virus. The cell-to-cell movement continued as normal in the inoculated leaf. There have been a number of reports of delayed systemic symptoms after inoculation of CPMR transgenic plants with the challenge virus. For example, transgenic tobacco plants expressing the CP gene of PRV showed a delay in systemic virus infection (Ling *et al.*, 1991). Clark *et al.* (1990) confirmed that the presence of transgenic CP in the phloem and associated cells of the plant interferes with the systemic spread of the virus. It is clear that there are many unanswered questions about the exact mechanisms surrounding CPMR and that the literature on this topic covers research experiments that each provides a small contribution to the understanding of CPMR. It is however by no means a universal mechanism operating in the same way for each virus.

Even though CPMR has been the most extensively applied, it is worth briefly mentioning some of the other forms of PDR. The initial demonstration that other nonstructural genes or genome fragments could be used to provide protection came from experiments which studied the involvement of a 54 kDa

read-through part of the replicase protein of TMV in replication (Golemboski *et al.*, 1990). The transgenic plants surprisingly showed a very high level of resistance to TMV infection and unlike CPMR, the protection was not overcome with very high levels of virus inoculum. There have been several reports of replicase-mediated resistance to different viruses, for example: pea early browning virus (PEBV) (MacFarlane & Davies, 1992), and cucumber mosaic virus (CMV) (Anderson *et al.*, 1992). These transgenic plants express either the entire or a mutated form of the replicase gene. The resistance tends to be strain specific and gives virtual immunity to virus infection. Studies on the mechanism of replicase-mediated resistance have shown that the expression of a transgenic replicase protein severely reduces virus replication in transgenic plants. Replication is impeded such that no systemic spread of the virus can occur (Carr & Zaitlin, 1991, Carr *et al.*, 1992).

Movement protein-mediated resistance is another form of PDR. Resistance is conferred by the transgenic expression of a dysfunctional MP, which in turn modifies the plasmodesmata and limits the cell to cell movement of the virus (Lapidot *et al.*, 1993). Generally, movement protein-mediated resistance is a "broad" resistance, which is overcome with high doses of virus inoculum (Beck *et al.*, 1994).

Resistance conferred by a transcript of a viral transgene and not the protein product include, satellite RNA-mediated resistance (Yie *et al.*, 1992), defective interfering RNA molecules (Kollàr *et al.*, 1993, Rubio *et al.*, 1999) and defective interfering DNA molecules (Stanley *et al.*, 1990).

Antisense RNA in its simplest form can be produced by inverting a cDNA copy of a mRNA with respect to the promotor in an expression vector. This will be transcribed as the complement of the mRNA sequence. Several experiments have transformed plants with constructs hosting the CP gene in the antisense orientation. For example: transgenic tobacco plants conferred resistance to TMV (Powell *et al.*, 1989) and transgenic potato plants conferred resistance to the potato leafroll virus (PLRV) (Palucha *et al.*, 1998). The antisense RNA-mediated resistance was shown to be weaker than CPMR and like CPMR the

resistance was largely overcome with an increased virus inoculum. Several experiments reporting antisense RNA-mediated resistance in transgenic plants have observed that the resistance conferred is very high, but that it is specific to a particular virus strain (Kawchuk *et al.*, 1991; Lindbo & Dougherty, 1992). It is thought that the antisense RNA complementary to the CP mRNA interferes with viral replication or translation by hybridizing to the sense genomic RNA (or DNA) or by competing for viral or host components needed for replication (reviewed in Hackland *et al.*, 1994).

RNA-mediated resistance does not require a functional protein such as in CPMR, but instead requires the accumulation of nucleic acid to provide protection. The expression of the transgene RNA alone is sufficient to provide resistance. This form of resistance has been observed on a number of occasions involving the RNA sequences of the replicase, MP and CP genes. For example: the expression of either a portion of, or the full-length replicase gene RNA sequences alone, conferred resistance in transgenic plants to PVX (Braun & Hemenway, 1992, Longstaff *et al.*, 1993). Also, the expression of an untranslatable CP gene RNA sequence was sufficient in providing resistance to PVY in transgenic plants (Smith *et al.*, 1994). Also, untranslatable transcripts of the MP gene was sufficient in providing resistance to the tomato spotted wilt virus (TSWV) in transgenic plants, thus suggesting RNA-mediated resistance (Prins *et al.*, 1996). From these and many other studies, it was clear that there was a lack of correlation between the level of expression of the transgenic protein and the level of resistance. Unlike CPMR, RNA-mediated resistance, as seen in the studies on TSWV, does not depend on the "dose" of virus inoculum and therefore provides complete immunity. In most cases of CPMR, the resistance decreases as the virus inoculum increases. Another difference is that RNA-mediated resistance was shown to be "narrow" and therefore specific to the virus from which the transgene was derived. With CPMR, as has been mentioned, resistance can be either "narrow" or "broad". It also became apparent in the studies on TSWV that the RNA-mediated resistance showed a negative or inverse correlation between the level of expression of RNA and the level of the resistance. In other words, the TSWV resistant plants showed a low level of RNA expression. This same

correlation was also shown in transgenic plants resistant to PVY (Smith *et al.*, 1994). It also became apparent that RNA-mediated resistance is not a form of antisense RNA-mediated resistance and operates in a different manner. The mechanism of RNA-mediated resistance is extremely complex, but it is thought to be closely related to the post-transcriptional gene-silencing phenomenon. This type of resistance is also referred to as homology-dependent resistance (Dougherty *et al.*, 1994, Prins & Goldbach, 1996, Wassenegger & Pélissier, 1998).

It is clear that PDR has become a very feasible approach, especially in crop plants where no naturally occurring resistance genes have been identified. The existing sanitation protocols are preventative measures and do not offer any long-term solution. The concept of PDR has been one of the greatest biotechnological breakthroughs and will certainly provide long-term solutions to pathogen problems. There is no limit to the hosts that can be exploited for the purpose of PDR, provided that it is technically feasible to be genetically modified in an ethically acceptable way.

1.8 PROGRESS IN THE DEVELOPMENT OF PDR IN GRAPEVINE

Transforming grapevine is a daunting, time-consuming task and there have been a number of failed attempts due to plant regeneration problems (Guellec *et al.*, 1990, Berres *et al.*, 1992). However, thanks to improved protocols, there have been numerous reports on the transformation of grapevine. Most of these transformations use *Agrobacterium tumefaciens* as a vector (Martinelli & Mandolino, 1994, Perl *et al.*, 1996, Xue *et al.*, 1999), however there has also been success with the biolistic transformation method (Kikkert *et al.*, 1996). Regardless of which method researchers choose to use, the ultimate goal is to transform grapevine with a virus-derived gene, such as the CP gene, to generate virus resistant cultivars. Transforming tobacco with the relevant gene usually precedes this. Tobacco has become the model plant for *Agrobacterium*-mediated transformations (reviewed in Hackland *et al.*, 1994). Tobacco is susceptible to a vast majority of plant viruses (GLRaV-1 &

GLRaV-3 to -8 are the exceptions) and several fast and reproducible tobacco transformation protocols exist. It is thus a quick, usually reliable method to test if the transgene is being expressed before pursuing grapevine transformation experiments. There have been promising results in engineering resistance to grapevine viruses. Some of the viruses concerned will be mentioned.

Grapevine chrome mosaic nepovirus (GCMV): *Nicotiana tabacum* tobacco plants were transformed with the CP gene of GCMV (Brault *et al.*, 1993). Leaf discs were infected with *A. tumefacien*s harboring a binary plant expression vector. This construct carried the GCMV CP gene, a cauliflower mosaic virus (CaMV) 35S promotor, a nopaline synthase (NOS) terminator and the kanamycin (Km) resistance, hygromycin resistance and β-glucuronidase (GUS) selectable markers. Transgenic plants were selected on Km and analyzed for the expression of the CP gene. It was shown that the CP gene was expressed in transgenic plants and the greater the level of expression, the greater the resistance. Resistance to GCMV was also characterized by the inhibition of virus accumulation in infected transgenic plants. Transgenic plants also conferred resistance to GCMV uncoated viral mRNA, which suggests the possibility of the involvement of other resistance mechanisms. Further research would have to explore these possibilities.

GVA and GVB: *Nicotiana benthamiana* and *Nicotiana occidentalis* tobacco plants were transformed with binary plant expression vectors harboring the CP genes of GVA and GVB respectively (Minafra *et al.*, 1998). Each construct was mobilized into *A. tumefaciens* and used to infect leaf discs. Each binary plant expression vector contained a CaMV 35S promotor, a NOS terminator and a Km resistance selectable marker. The transgenic plants were selected on Km and analyzed for the expression of the CP gene. Most transgenic *N. benthamiana* and *N. occidentalis* plants were shown to express the CP genes of GVA and GVB respectively, however, there was no correlation between the amount of CP expressed and the level of resistance. Some GVA and GVB transgenic plants that did not express CP were still

resistant to the respective virus, which indicated that the mRNA was sufficient to provide resistance. Infected transgenic plants showed a decrease in virus accumulation associated with a delayed systemic virus infection. These same two binary plant expression vectors were used to transform somatic embyogenic tissue of *Vitis vinifera* (Gölles *et al.*, 2000). Transgenic plants were selected on Km for ~1 year. These grapevines were shown to express the CP genes of GVA and GVB respectively, however the plants are still undergoing resistance evaluation. *N. benthamiana* and *N. occidentalis* tobacco plants were also transformed with binary plant expression vectors harboring the MP genes of GVA and GVB respectively (Buzkan *et al.*, 2000). The MP gene of each virus was inserted in both sense (GVA+, GVB+) and antisense (GVA-, GVB-) orientations. Each construct was mobilized into *A. tumefaciens* and used to infect leaf discs. Transgenic plants were selected on Km and tested for the presence of the MP gene. When GVA+ transgenic plants were challenged with GVA, a high percentage of the plants still showed symptoms. When GVA- transgenic plants were challenged with GVA, ~40 % of all the plants showed a reduced virus replication rate and were symptomless. When GVB+ transgenic plants were challenged with GVB, a high percentage of the plants still showed symptoms but a lower level of virus accumulation accompanied this. When GVB- transgenic plants were challenged with GVB, only very few of the plants were symptomless. From this it is clear that the transgenic plants show various degrees of resistance to these viruses. Even though these results are for the most part "chaotic", the fact that some plants are resistant to these viruses is certainly a good starting point for further research. These same constructs harboring the MP genes (sense and antisense) of GVA and GVB respectively, were used to transform somatic embryos of *Vitis rupestris* and embriogenic calli of *Vitis vinifera* via *A. tumefaciens* (Buzkan *et al.*, 2000). Regenerated grapevines are in the process of being evaluated for resistance to GVA and GVB.

GLRaV-2: *N. Benthamiana* tobacco plants were transformed with the CP gene of GLRaV-2 (Gonsalves, 2000). Transgenic plants were shown to express the CP gene. Since GLRaV-2 is the only GLRaV that can be transmitted to herbaceous plants, the transgenic plants were challenged with the virus. The

transgenic plants were resistant to GLRaV-2, however, there was no correlation between expression of the CP gene and the resistance, suggesting RNA-mediated resistance. Grapevine has also been transformed with the CP gene of GLRaV-2, but these plants are also in the process of being evaluated for resistance (Gonsalves, 2000).

GLRaV-3: N. Benthamiana tobacco plants were transformed with binary plant expression vectors harboring the CP gene of GLRaV-3 in both the sense and antisense orientations (Ling *et al.*, 1997). Each construct was mobilized into *A. tumefaciens* and used to infect leaf discs. Both sense and antisense binary plant expression vectors contained a double enhanced CaMV 35S promotor and terminator, an alfalfa mosaic (ALMV) virus RNA 5' leader sequence and a Km resistance gene. Transgenic plants were selected on Km and tested for the presence of the CP gene. The transgenic plants, in which successful insertion of the CP gene took place, were tested for CP expression. As expected, the transgenic plants with the CP gene in the sense orientation showed CP expression, whereas those with the CP gene in the antisense orientation showed no CP expression. As mentioned before, GLRaV-3 can not be transmitted to herbaceous species and therefore resistance in tobacco can not be determined. These transformation experiments therefore only proved the expression of the CP gene and ultimately the functionality of the plant expression construct. These same constructs harboring the CP gene (sense and antisense) of GLRaV-3 as well as a binary plant expression vector harboring a truncated HSP90h 43 kDa gene (Ling *et al.*, 1998) were used to transform somatic embryos of grapevine rootstocks (*Vitis* spp.) (Xue *et al.*, 1999, Gonsalves, 2000). Regenerated plants were selected on Km and tested for the presence of these genes. Transgenic grapevines were shown to contain these genes, but again these plants are still in the process of being evaluated for resistance to GLRaV-3.

GFLV: *N. Benthamiana* tobacco plants were transformed with the CP gene of GFLV (Gölles *et al.*, 2000). A number of binary plant expression vectors harboring the GFLV CP gene were constructed. These were as follows: one carried the full-length CP gene, another differed from this one by a 15 base

pair (bp) deletion within the gene. One carried an untranslatable CP gene in the sense orientation; another carried an untranslatable CP gene in the antisense orientation. One carried the CP gene with a truncation at the 5' end; another carried the CP gene with a truncation at the 3' end. Each of these constructs contained a CaMV 35S promotor, a NOS terminator and a Km resistance gene. *A. tumefaciens* was used to infect leaf discs. Transgenic plants were selected on Km and tested for the presence on the GFLV CP gene. The transgenic tobacco plants were totally resistant to GFLV. These same constructs were used to transform somatic embryogenic tissue of *Vitis vinifera* via *A. tumefaciens*. Regenerated plants were also selected on Km for ~1 year. The transgenic plants were tested for the presence of the CP gene. Serologically, no CP could be detected in those plants that contained the full-length CP gene. Furthermore, another research group have transformed grapevine with the GFLV CP gene (Fuchs *et al.*, 2000). Analysis of CP expression in transgenic plants has revealed varying expression levels, from totally undetectable to very high. Transgenic grapevine has apparently shown very promising levels of GFLV resistance after a four-year field trial (Fuchs *et al.*, 2000).

1.9 PROJECT OUTLINE AND AIMS

The aims of this project are:

- To isolate and clone the CP gene of a South African isolate of GLRaV-3 from leafroll infected grapevines
- To sequence the GLRaV-3 CP gene and compare the data to the only other existing GLRaV-3 CP gene sequence (Ling *et al.*, 1997)
- To subclone the GLRaV-3 CP gene into plant expression vectors in the sense and antisense orientations
- To use these recombinant plant expression vectors for the transformation of tobacco and subsequently grapevine (envisaged by the Institute for Wine Biotechnology, Stellenbosch)
- To determine whether the GLRaV-3 CP gene (sense and antisense) is expressed in tobacco

The project was motivated because of the serious problem of grapevine leafroll disease in South African vineyards. It is a starting point towards the ultimate goal of introducing resistance in grapevine to leafroll disease.

The isolation and cloning of the GLRaV-3 CP gene was motivated because: first, the CP gene is a good source of a virus-derived gene for the prospect of pathogen-derived resistance and the sequence thereof recently became available (Ling *et al.*, 1997). Second, the serious problem of re-infection of leafroll in South African vineyards (Kriel, pers. comm.) certainly warrants research on virus-derived genes as possible sources for PDR. As mentioned before, PDR can provide a long-term solution for the virus problem. Third, previous attempts to isolate and clone the GLRaV-3 CP gene in South Africa have failed.

The motivation for cloning the GLRaV-3 CP gene in the sense and antisense orientations into plant expression vectors is to determine in subsequent grapevine transformation experiments, whether the resistance conferred, if any, is CPMR, RNA-mediated resistance or antisense RNA-mediated resistance.

CHAPTER 2

THE ISOLATION, CLONING AND MOLECULAR CHARACTERIZATION OF THE COAT PROTEIN GENE OF A SOUTH AFRICAN ISOLATE OF GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS-3

SUMMARY

The CP gene of a South African isolate of GLRaV-3 infected grapevine was isolated, cloned and sequenced. Double stranded RNA was extracted from GLRaV-3 infected material and a high molecular weight band, of ~18 kb was consistently identified from infected vines and not from healthy vines. The dsRNA was used as a template in reverse transcription PCR together with GLRaV-3 CP gene specific primers for the amplification of the GLRaV-3 CP gene. The amplification product was the correct size (975 bp), corresponding to the GLRaV-3 CP gene and flanking nucleotides derived from the GLRaV-3 CP gene specific primers. The PCR product was cloned into the pGem®-T Easy vector system and restriction enzyme analysis was used to select two clones, one with the insert in the sense orientation (pLR3CP+) and one with the insert in the antisense (pLR3CP-) orientation. These two clones were sequenced and the data obtained analyzed. The sequence showed 99.26 % similarity to the only other GLRaV-3 CP nucleotide sequence available.

2.1 INTRODUCTION

The molecular characterization of GLRaV-3 and the other GLRaVs have been difficult, primarily due to difficulties in virus purification methods. Grapevine plant material contains phenolic compounds, which inhibit the extraction of nucleoproteins (Loomis, 1974, Flores *et al.*, 1985). The phloem-bound nature of GLRaV particles and the occurrence of mixed infections in grapevine have contributed to the many failed attempts of purifying sufficient amounts of pure virus isolates (Choueiri *et al.*, 1996). Extensive serological studies characterized GLRaV-3 from the other GLRaVs, which facilitated the molecular characterization of GLRaV-3 (Hu *et al.*, 1990a, Zimmermann *et al.*, 1990). The extraction and analysis of dsRNA proved to be an alternative method in detecting closteroviruses from leafroll infected vines (Mossop *et al.*, 1985, Rezaian *et al.*, 1991, Habili & Rezaian, 1995). The dsRNA is the replicative form of genomic RNA (Dodds & Bar-Joseph, 1983). It is very stable and is resistant to DNase as well as to RNase in high salt concentrations (Hu *et al.*, 1990b). The presence of a high Mr dsRNA of ~18 kb was consistently detected from GLRaV-3 infected vines, as determined by serological assays (Hu *et al.*, 1990b, Salderelli *et al.*, 1994, Habili *et al.*, 1995). The use of dsRNA from leafroll infected vines as a template in the production of cDNA clones was reported by Rezaian *et al.* (1991). The dsRNA of GLRaV-3 was later used as a template in the production of a cDNA library by a process known as reverse transcription polymerase chain reaction (RT-PCR) (Ling *et al.*, 1997). This cDNA library was immunoscreened with GLRaV-3 specific monoclonal and polyclonal antibodies. The GLRaV-3 CP gene with an electrophoretic estimated size of ~43 kDa, was subsequently isolated, cloned and sequenced.

This chapter reports the extraction of dsRNA from South African GLRaV-3 infected vines and the subsequent molecular cloning and nucleotide sequencing of the CP gene. The strategies used for cloning the CP gene and selecting clones with the CP gene in the sense and antisense orientations are discussed. The nucleotide sequence obtained from the South African

GLRaV-3 CP gene is compared with the only other nucleotide sequence of the GLRaV-3 CP gene (Ling *et al.*, 1997).

2.2 MATERIALS AND METHODS

2.2.1 *Extraction and analysis of dsRNA*

GLRaV-3 infected leaf and bark tissue from the rootstock variety, LN33 (vine no. 1/2/5) was collected from vines grown at the Nietvoorbij experimental farm of the Agricultural Research Council. This material was indexed for the presence of viruses by immunoelectron miscroscopy (Kasdorf, pers. comm.) and ELISA (Carstens, pers. comm.). Both tests indicated the presence of GLRaV-3 only.

Veins of the leaf or cortex tissue of the bark were used to extract viral dsRNA according to the method by Rezaian *et al.* (1991) with minor modifications. Approximately 15 g each of infected and healthy material was ground in liquid nitrogen and mixed with extraction media (4 ml/g), containing 200 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 3 % SDS, 10 % ethanol and 1% 2-mercaptoethanol. Samples were shaken gently at 37 °C for 10 minutes and extracted with ½ volume of chloroform by shaking again for 30 minutes at room temperature, followed by centrifugation at 10 000 g for 10 minutes. The supernatant was mixed with 2 g of Whatmann CF11 cellulose (Merck) and ethanol was added to a final concentration of 17 %, disregarding the ethanol in the extraction media. The mixture was poured in a column, allowed to run dry and washed three times with 15 ml 17 % ethanol in 1x STE (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). The dsRNA was eluted with 10 ml 1x STE and mixed with 0.2 g CF11. The mixture was poured into a column, allowed to dry and washed three times as before. The dsRNA was eluted with 1 ml of 1x STE and precipitated overnight at –20 °C with 2.5 volumes ethanol and 1/3 volume 3 M sodium acetate, pH 5.2. The dsRNA was recovered by centrifugation at 6 000 g for 30 minutes. The pellet was resuspended in

500 µl 1x STE and precipitated as before for 4 hours at –70 ºC. Pellets of dsRNA were recovered again by centrifugation at 6 000 g for 60 minutes and resuspended in 100 µl sterile H_2O. Samples were analyzed on a 1 % low melting point agarose gel in a TAE buffer system and visualized with ethidium bromide staining. A 23 kb *Hind* III digested lambda DNA ladder (Promega) was used as a standard to determine the size of the dsRNA fragment.

2.2.2 *Oligonucleotide primer sequences, viral cDNA synthesis and analysis of PCR products*

Three oligonucleotide primers were designed by computer analysis (Primer3 output, (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) using the published GLRaV-3 CP gene nucleotide sequence (Ling *et al.*, 1997). The 5' primer (LR3-CP02-For: GATCTAGACC**ATGGCATTTGAACTGAAA)** and the 3' primer (LR3-CP02-Rev: GTTCTAGAGGTCACCGATCGTAG**CTACTT**) flank the GLRaV-3 CP gene. LR3-CP02-For contains two restriction enzyme (RE) sites, *Xba* I (red) and *Nco* I (underlined), and a start codon. LR3-CP02-Rev contains two RE sites, *Xba* I (red) and *BstE* II (underlined), and a stop codon. The bold sections represent nts from the GLRaV-3 CP gene and the section in green represents nts flanking the GLRaV-3 CP gene i.e. from the GLRaV-3 genome. The RE sites were incorporated to facilitate subcloning the GLRaV-3 CP gene. A third primer, which will be referred to as the "cDNA primer" (LR3-CP01-Rev: ATCGATCGTAG**CTACTTCTTTTGC**) was synthesized without RE sites for use in viral cDNA synthesis. First strand cDNA synthesis was performed using recombinant Moloney Murine Leukemia Virus reverse transciptase (SUPERSCRIPT™ II; GibcoBRL, Life Technologies) according to the protocol supplied by the manufacturer. In a PCR tube, 10 µl of dsRNA in H_2O and 1 µl of "cDNA primer" were added together and incubated at 96 ºC for 5 minutes to denature the dsRNA. The reaction was rapidly cooled on ice to induce primer annealing. The reverse transcriptase mixture calculated for a final reaction volume of 20 µl contained 4 µl 5x first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl 100 mM DTT, 1 µl 10 mM deoxynucleoside triphosphates (dNTP) mix and 40 U Rnasin (Promega). The

reverse transcriptase mixture was added to the RNA sample and incubated at 42 °C for 2 minutes, after which 200 U (10 U/µl) SUPERSCRIPT™ II reverse transcriptase was added and incubated at 42 °C for 1 hour. The reaction was inactivated at 70 °C for 15 minutes. One microliter of various dilutions of the cDNA was used as a template for the amplification of the GLRaV-3 CP gene in a PCR reaction as described in Appendix A.1 with the GLRaV-3 CP gene specific primers, LR3-CP02-For and LR3-CP02-Rev. The PCR products were analyzed on a 1.4 % TAE agarose gel. The 1 kb plus DNA ladder (GibcoBRL, Life Technologies) consisting of DNA fragments ranging in size from 100 to 12 000 bp was used to determine the size of the amplified DNA products.

2.2.3 *Cloning the PCR product, restriction enzyme and sequence analysis*

The PCR products of correct size (975 bp) were purified from a 0.8 % TAE agarose gel using the Qiagen gel extraction kit (Southern Cross Biotechnology) and the concentration was determined on a spectrophotometer. All subsequent concentrations were also determined on a spectrophotometer. The PCR product was then ligated into the 3018 bp pGem®-T Easy Vector (Promega) (Fig. 2.1) according to the manufacturer's instructions and transformed into *Escherichia coli* strain DH5α. Clones were selected for the insert by blue/white colour screening. Recombinant clones were used to inoculate 5 ml Luria-Bertani (LB) medium containing the antibiotic, ampicillin at a concentration of 100 µg/ml (Amp^{100}). The isolation of plasmid DNA from overnight bacterial cultures was performed by using the ABI Prism™ Miniprep Kit (Perkin Elmer Applied Biosystems) according to the manufacturer's instructions. PCR and size analysis on a 1.4 % TAE agarose gel was used to confirm the presence of the GLRaV-3 CP gene. Digestion of the recombinant clones with the RE, *Nco* I (Appendix A.2) determined the orientation to be either sense (pLR3CP+) or antisense (pLR3CP-) according to the size of the digested fragments. Both pLR3CP+ and pLR3CP- were sequenced in an ABI 377 automated DNA sequencer (University of Stellenbosch Core Sequencing Facility) using T7/Sp6 sequencing primers and standard protocols from the supplier.

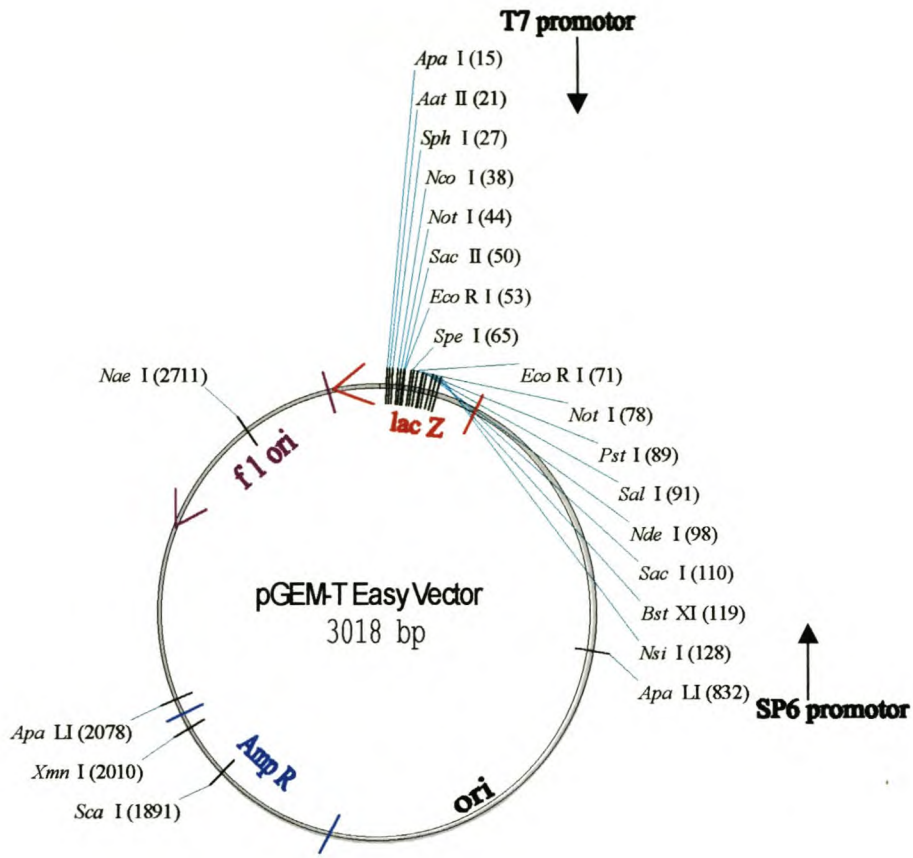

Fig. 2.1. Physical map of pGEM®-T Easy Vector (courtesy of Promega).

2.3 RESULTS AND DISCUSSION

2.3.1 *Extraction of dsRNA and analysis of PCR products*

Viral dsRNA was successfully extracted from bark cortex tissue of GLRaV-3 infected vines and not from healthy control vines. The purified dsRNA showed a high Mr band of ~18 kb, associated with GLRaV-3, as had been previously observed (Hu *et al.*, 1990b, Ling *et al.*, 1997) (Fig. 2.2). Although the yield was very low, the dsRNA was used in a RT-PCR reaction with the GLRaV-3 CP gene specific primers, LR3-CP02-For and LR3-CP02-Rev. One microliter of undiluted and 1 µl of diluted (1/10, 1/100 and 1/1000) synthesized first strand cDNA used as templates in PCR consistently showed an amplification product of 975 bp. This fragment includes the 942 bp GLRaV-3 CP gene, which has been previously identified (Ling *et al.*, 1997) and an

additional 33 nts derived from the incorporation of RE sites from the primers, LR3-CP02-For and LR3-CP02-Rev (Fig. 2.3).

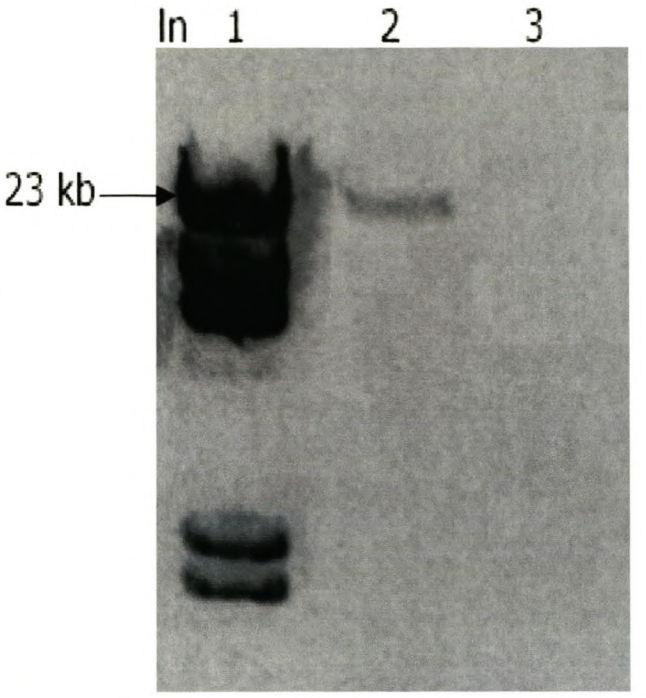

Fig. 2.2. Size determination of GLRaV-3 dsRNA on a 0.8 % TAE low melting point agarose gel. Lane 1, 23 kb *Hind* III digested lambda DNA ladder. Lane 2, a dsRNA band of ~18 kb was derived from GLRaV-3 infected material. Lane 3, the healthy control plant did not show any dsRNA band.

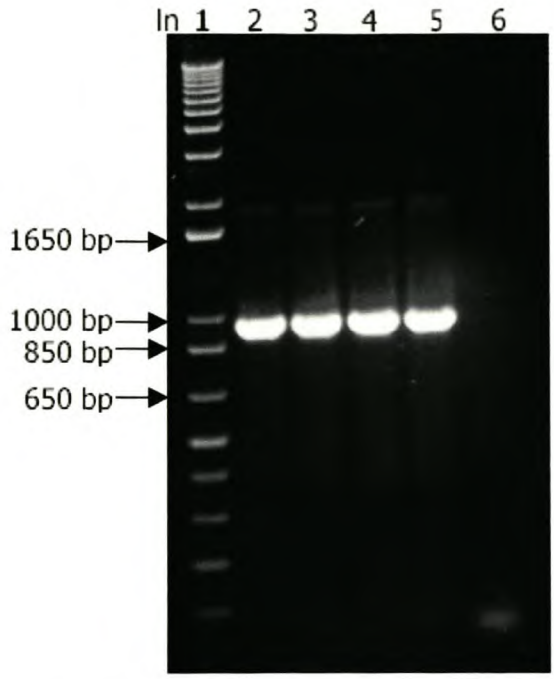

Fig. 2.3. RT-PCR amplication products (using GLRaV-3 CP gene specific primers) on a 1.4 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lane 2, undiluted cDNA used as a template in RT-PCR produced the fragment of correct size i.e. 975 bp, representing the GLRaV-3 CP gene and flanking nts derived from the GLRaV-3 CP gene specific primers. Lane 3-5, a diluted series of cDNA (1/10, 1/100 and 1/1000 respectively) was used successfully to amplify the same product. Lane 6, H_2O was used in a reaction as a negative control.

2.3.2 *Cloning the PCR product, restriction enzyme and sequence analysis*

The PCR product was ligated into the 3018 bp pGem®-T Easy Vector. Four white colonies and one blue colony were screened for the presence of the GLRaV-3 CP gene. PCR analysis using the GLRaV-3 CP gene specific primers showed that a 975 bp fragment was consistently amplified from the white colonies and not from the blue colony (results not shown). The plasmid DNA of all 5 colonies was linearized with *Pst* I and gel electrophoresed for size analysis. The four white colonies (clones 1-4) contained the insert (3993 bp) and the blue colony (clone 5) did not contain the insert (3018 bp) (Fig. 2.4).

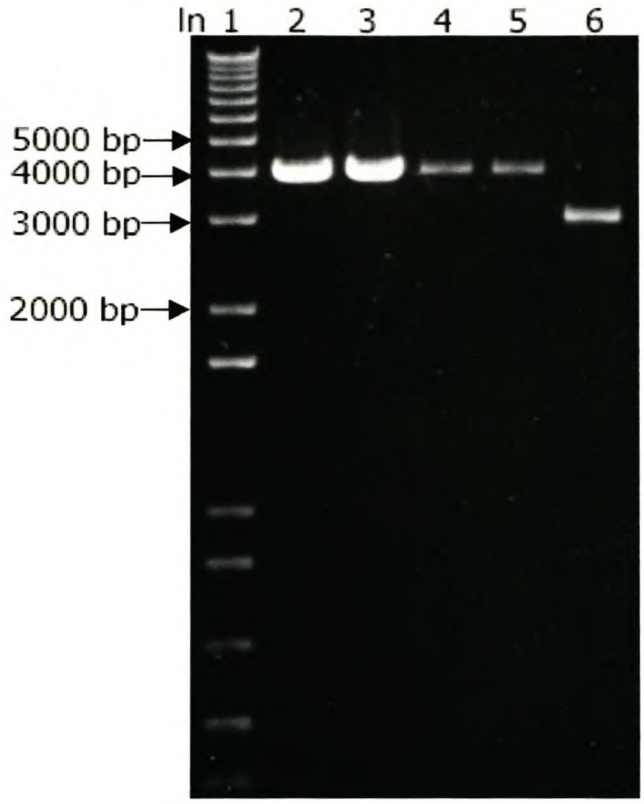

Fig. 2.4. Size analysis of pGem®-T Easy Vector with and without the insert on a 1.4 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lane 2-5, the 4 white colonies (clones 1-4) are each 3993 bp in size and therefore all contain the 975 bp GLRaV-3 CP gene insert. Lane 6, the blue colony (clone 5) is the same size as the pGem®-T Easy Vector i.e. 3018 bp and therefore does not contain the insert.

Clones 1-4 were digested with *Nco* I to determine the orientation of the GLRaV-3 CP gene insert. Of the four clones tested, one had the GLRaV-3 CP gene insert ligated in the antisense orientation. As depicted in Fig. 2.5.1, if the gene was to be inserted in the sense orientation and the resultant vector digested with *Nco* I, two fragments of sizes 3960 bp and 33 bp will be produced. If the gene was to be inserted in the antisense orientation (Fig. 2.5.2) and the resultant vector digested with *Nco* I, two fragments of sizes 3003 bp and 990 bp will be produced.

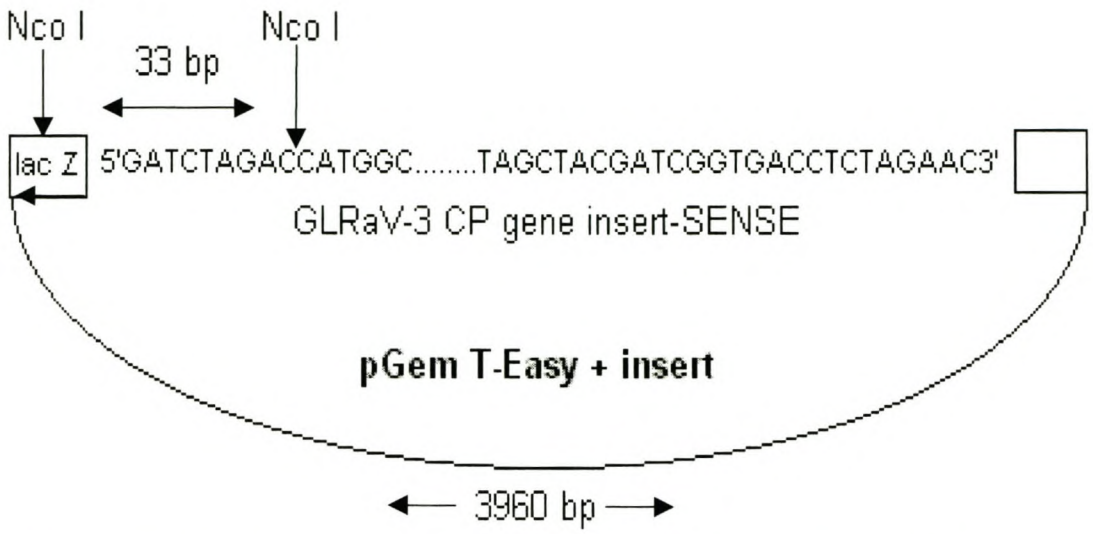

Fig. 2.5.1. Diagrammatic representation of the GLRaV-3 CP gene ligated in the sense orientation. Digestion of this clone with *Nco* I would result in two fragments, one of 3960 bp and one of 33 bp.

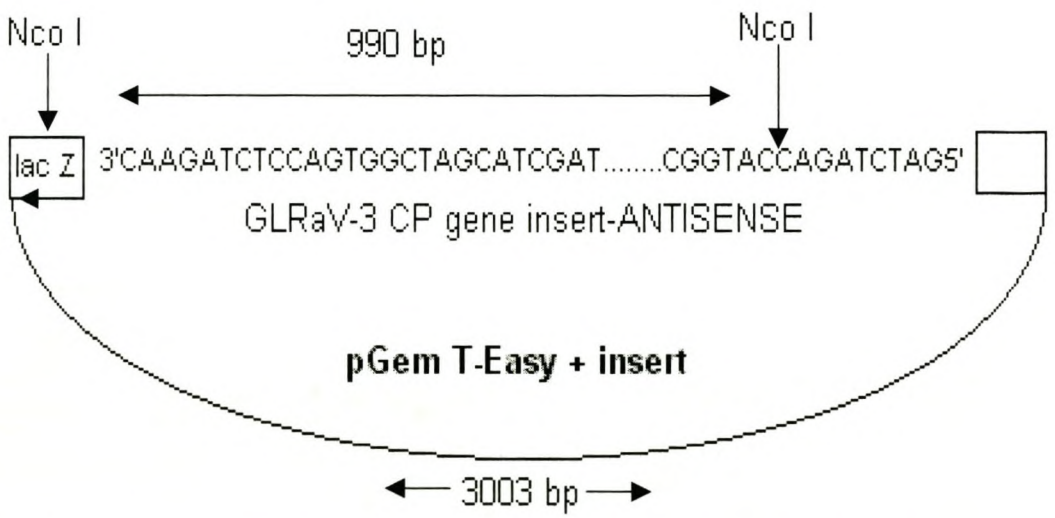

Fig. 2.5.2. Diagrammatic representation of the GLRaV-3 CP gene ligated in the antisense orientation. Digestion of this clone with *Nco* I would result in two fragments, one of 3003 bp and one of 990 bp.

The clones with the gene in the sense orientation clearly showed a 3960 bp fragment, but the 33 bp fragment could not be seen on the 1.4 % TAE agarose gel, as it had run off. Also, the clone with the gene in the antisense direction clearly showed two bands of the correct size (Fig. 2.6). One sense clone was selected and designated as pLR3CP+ and the antisense clone was designated pLR3CP-. These two clones were sequenced and the data obtained (Appendix B.1) showed 99.26 % similarity to the nt sequence of the GLRaV-3 CP gene characterized by Ling *et al.* (1997).

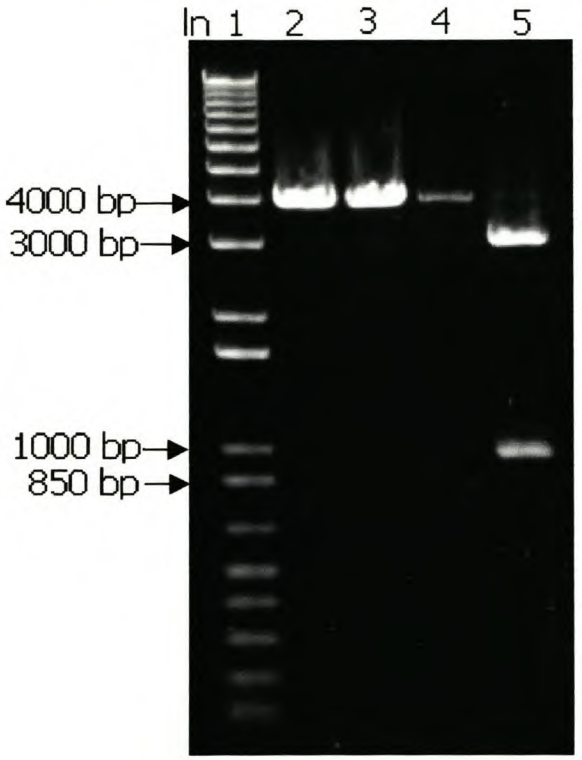

Fig. 2.6. Determining the orientation of the GLRaV-3 CP gene by digesting clones 1-4 with *Nco* I and analyzing the products on a 1.4 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lane 2-4, clones 1-3 produced two fragments of sizes 3960 bp and 33 bp and thus has the GLRaV-3 gene fragment ligated in the sense orientation. The 3960 bp fragments can be seen clearly, but the 33 bp fragments have run off the end of the gel. Lane 5, clone 4 produced two fragments of sizes 3003 bp and 990 bp, which can both be seen clearly on the gel. Clone 4 thus has the GLRaV-3 CP gene ligated in the antisense orientation.

2.4 CONCLUDING REMARKS

The ~18 kb dsRNA derived from South African GLRaV-3 infected grapevine material was used as a template for the production of cDNA. The cDNA served as a template and together with GLRaV-3 CP gene specific primers were used for the amplification of a 975 bp fragment by PCR. The PCR product was cloned into the pGem®-T Easy vector system. This is a convenient system for cloning PCR products. The vector contains 3'-T overhangs for easy ligation of PCR products and there is convenient RE sites available to facilitate subcloning strategies. A number of white colonies were tested for the presence of the CP gene. Restriction enzyme size analysis was performed on these positive colonies to select two clones, one with the CP gene in the sense orientation and one with the CP gene in the antisense orientation. Only one of the four clones that were screened contained the CP gene in the antisense orientation. Our expectation that the CP gene had been isolated was confirmed by sequence analysis (Appendix B.1). An alignment between the South African GLRaV-3 CP gene and the published GLRaV-3 CP gene sequences (Ling *et al.*, 1997) was performed. There is a 99.26 % similarity between the South African GLRaV-3 CP gene and published GLRaV-3 CP gene sequences. There is a single base pair difference in 7 of the 942 nt positions, as indicated in Appendix B.1. In conclusion, the CP gene of a South African isolate of GLRaV-3 was isolated, cloned in the sense and antisense orientations and sequenced.

CHAPTER 3

SUBCLONING THE GRAPEVINE LEAFROLL ASSOCIATED CLOSTEROVIRUS-3 COAT PROTEIN GENE INTO PLANT EXPRESSION VECTORS IN BOTH SENSE AND ANTISENSE ORIENTATIONS

SUMMARY

The GLRaV-3 CP gene was excised from pLR3CP+ and pLR3CP- and subcloned into a plant expression vector, pCAMBIA 3301 in the sense and antisense orientations respectively. Subcloning the GLRaV-3 CP gene in the antisense orientation into pCAMBIA 3301 was facilitated by using an intermediate vector, pMECA. The resultant recombinant vectors hosting the GLRaV-3 CP gene in the sense and antisense orientations was designated as pCamBLR3CP+ and pCamBLR3CP- respectively. The GLRaV-3 CP gene was also subcloned from pCamBLR3CP+ into another plant expression vector, pCAMBIA 2301 in the sense orientation. During all subcloning steps the presence of the GLRaV-3 CP gene was confirmed by PCR with GLRaV-3 CP gene specific primers and restriction enzyme size analysis.

3.1 INTRODUCTION

A number of plasmid vectors termed "binary vectors" have been constructed for transferring useful genes via *Agrobacterium*-mediated transformation into the genomes of higher plants (Macbride & Summerfelt, 1990). In order for a binary vector to be useful it must possess the following: an antibiotic or herbicide resistance gene, which imparts to the plant cell the capability to grow in the presence of that antibiotic or herbicide. This is commonly referred to as a plant selectable marker. A widely used marker is the neomycin phosphotransferase II (NPT II) gene which imparts resistance to the antibiotic, kanamycin (Bevan *et al.*, 1983, Fraley *et al.*, 1983). The NPT II gene is derived from a bacterial transposable element and is therefore controlled by elements, which are bacterial in nature. It is therefore necessary to incorporate eukaryotic controlling elements or promoters to ensure gene expression. Two such widely used promotors are the NOS (An, 1986) and the CaMV 35S (Guilley *et al.*, 1982, Odell *et al.*, 1985) promotors. Another widely used plant selectable marker is the bialophos/Basta (*bar*) gene, which encodes the enzyme, phosphinothricin acetyl transferase (PAT) (De Block *et al.*, 1987). This enzyme detoxifies phosphinothricin (PPT), which is the active ingredient in the herbicide commercially known as Basta. The *bar* gene is derived from the bacterium *Streptomyces hygroscopicus* (White *et al.*, 1989) and therefore also needs to be driven by an eukaryotic promotor such as the those mentioned for the NPT II gene. The GUS reporter gene, the expression of which can be determined histochemically in plant cells, is also often incorporated in a binary vector under the control of a promotor (Jefferson *et al.*, 1987). The expression of the reporter gene verifies the insertion of the transfer DNA (T-DNA) region in putative transformants. The binary vector also needs a bacterial antibiotic resistance gene for selecting recombinant bacterial colonies. There are many examples such as the kanamycin, ampicillin, hygromycin and streptomycin resistance genes (reviewed in Svab *et al.*, 1990). There also needs to be RE sites in the portion of the binary vector that is transferred to the plant genome, in which to clone the gene of interest. It is imperative that the vector has one or both cis-active T-DNA border sequences. These are required for excising the DNA between the left

and right borders and then transferring the DNA from the *Agrobacterium* to the plant genome (Wang *et al.*, 1984).

This chapter reports the subcloning of the GLRaV-3 CP gene into two binary plant expression vectors, pCAMBIA 3301 and pCAMBIA 2301. The GLRaV-3 CP gene is subcloned in the sense orientation in pCAMBIA 3301 and pCAMBIA 2301. It is also subcloned in the antisense orientation in pCAMBIA 3301. The plasmid vector, pMECA contains a number of useful RE sites. Two of these, *Apa* I and *Sal* I are necessary for subcloning the GLRaV-3 CP gene in the antisense orientation into pCAMBIA 3301. This intermediate subcloning step is necessary to maintain the antisense orientation of the GLRaV-3 CP gene with regard to the CaMV 35S promotor of the plant expression vector.

3.2 MATERIALS AND METHODS

3.2.1 *Subcloning the GLRaV-3 CP gene in the sense orientation into pCAMBIA 3301*

Refer to Fig. 3.1 for a diagrammatic representation of the cloning strategy. The GLRaV-3 CP gene was excised from the sense construct, pLR3CP+ (refer to Fig. 2.4) by digestion with *Nco* I and *BstE* II. The digests were electrophoresed on a 1.4 % TAE agarose gel to separate the fragments. The 952 bp fragment corresponding to the GLRaV-3 CP gene and flanking RE site nts was cut from the gel, purified and the concentration determined. Simultaneously the 11 307 bp plant expression vector, pCAMBIA 3301 (CAMBIA, Australia) (Fig. 3.2) was digested with *Nco* I and *BstE* II and electrophoresed to separate the fragments. The 9258 bp fragment corresponding to the vector without the GUS reporter gene was cut from the gel, purified and the concentration determined. The 952 bp GLRaV-3 CP gene and 9258 bp pCAMBIA 3301 vector fragments were ligated in 30:1, 20:1, 10:1 and 3:1 insert to vector ratios (Appendix A.3) using the rapid

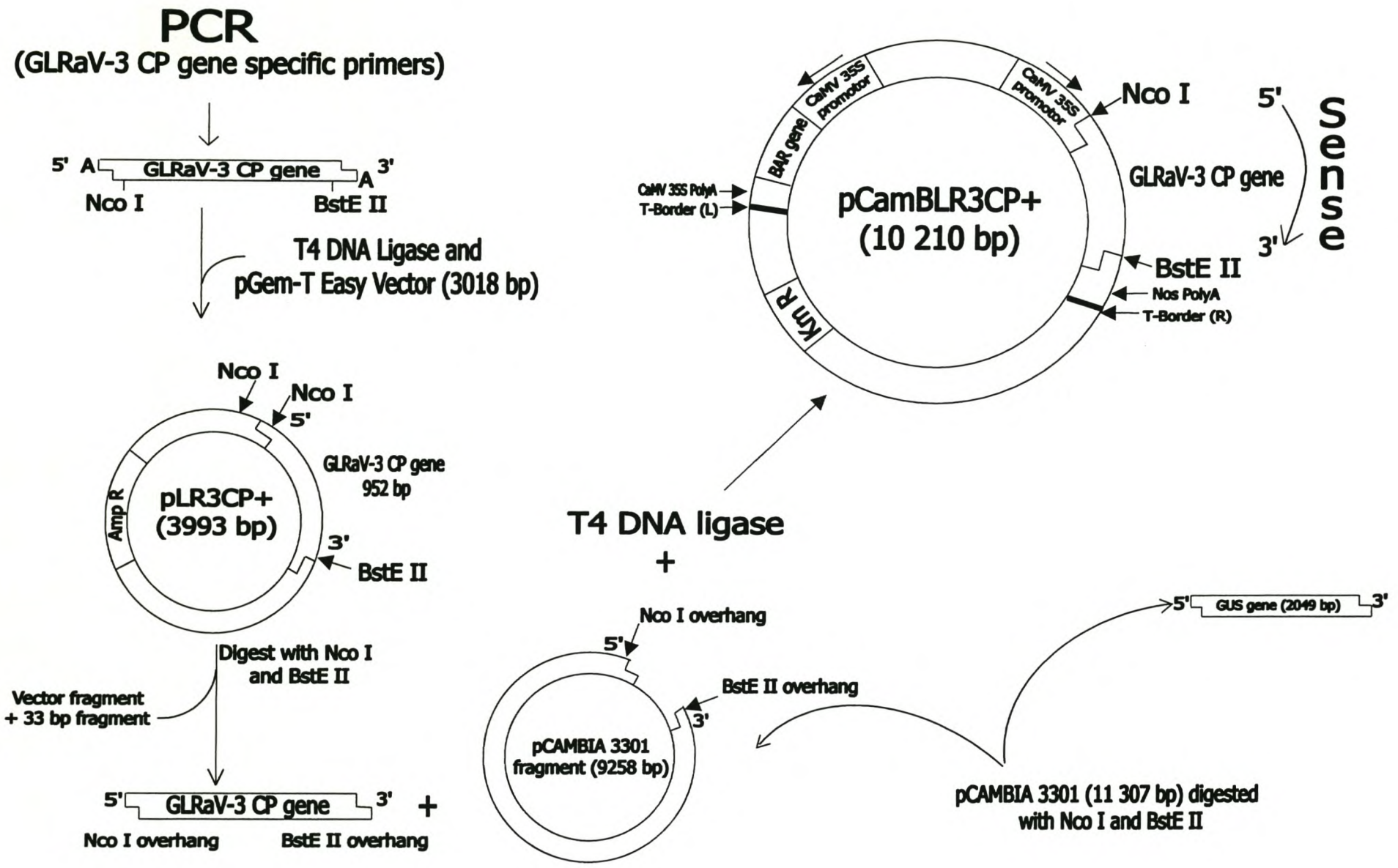

Fig. 3.1. Diagrammatic representation of subcloning the GLRaV-3 CP gene in the sense orientation into pCAMBIA 3301.

ligation kit (Roche Biochemicals) and transformed into *E. coli* strain DH5α. Colonies were selected for the insert on LB medium containing the antibiotic, kanamycin at a concentration of 100 µg/ml (Km^{100}). Plasmid DNA was isolated from overnight bacterial cultures as before. PCR and size analysis was used to confirm the presence of the GLRaV-3 CP gene. The recombinant pCAMBIA 3301 hosting the GLRaV-3 CP gene in the sense orientation was designated as pCamBLR3CP+. Glycerol stocks were made of pCamBLR3CP+ and stored at –80 °C.

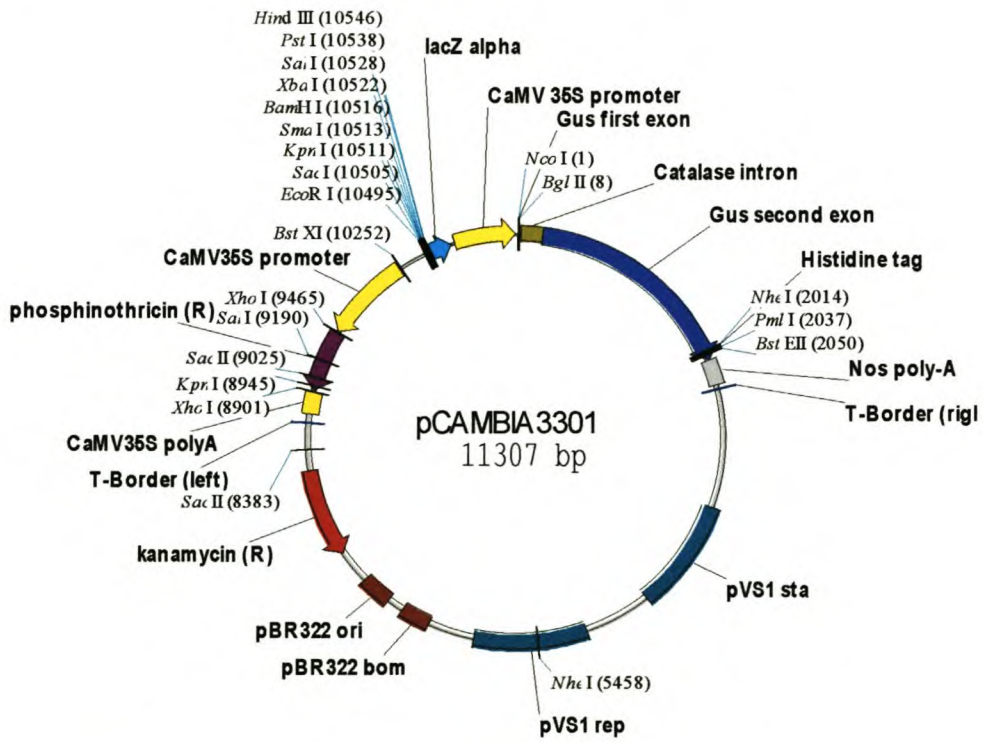

Fig. 3.2. Physical map of pCAMBIA 3301 (courtesy of CAMBIA, Australia).

3.2.2 *Subcloning the GLRaV-3 CP gene in the sense orientation into pCAMBIA 2301*

Refer to Fig. 3.3 for a diagrammatic representation of the cloning strategy. The GLRaV-3 CP gene was subcloned in the sense orientation into another plant expression vector, pCAMBIA 2301 (11 621 bp) (Fig. 3.4). The recombinant plasmid, pCamBLR3CP+ (10 210 bp) was digested with *BstE* II and *Pst* I and electrophoresed to separate the fragments. The 1721 bp fragment corresponding to the lac Z α-CaMV 35S promotor-GLRaV-3 CP gene was cut

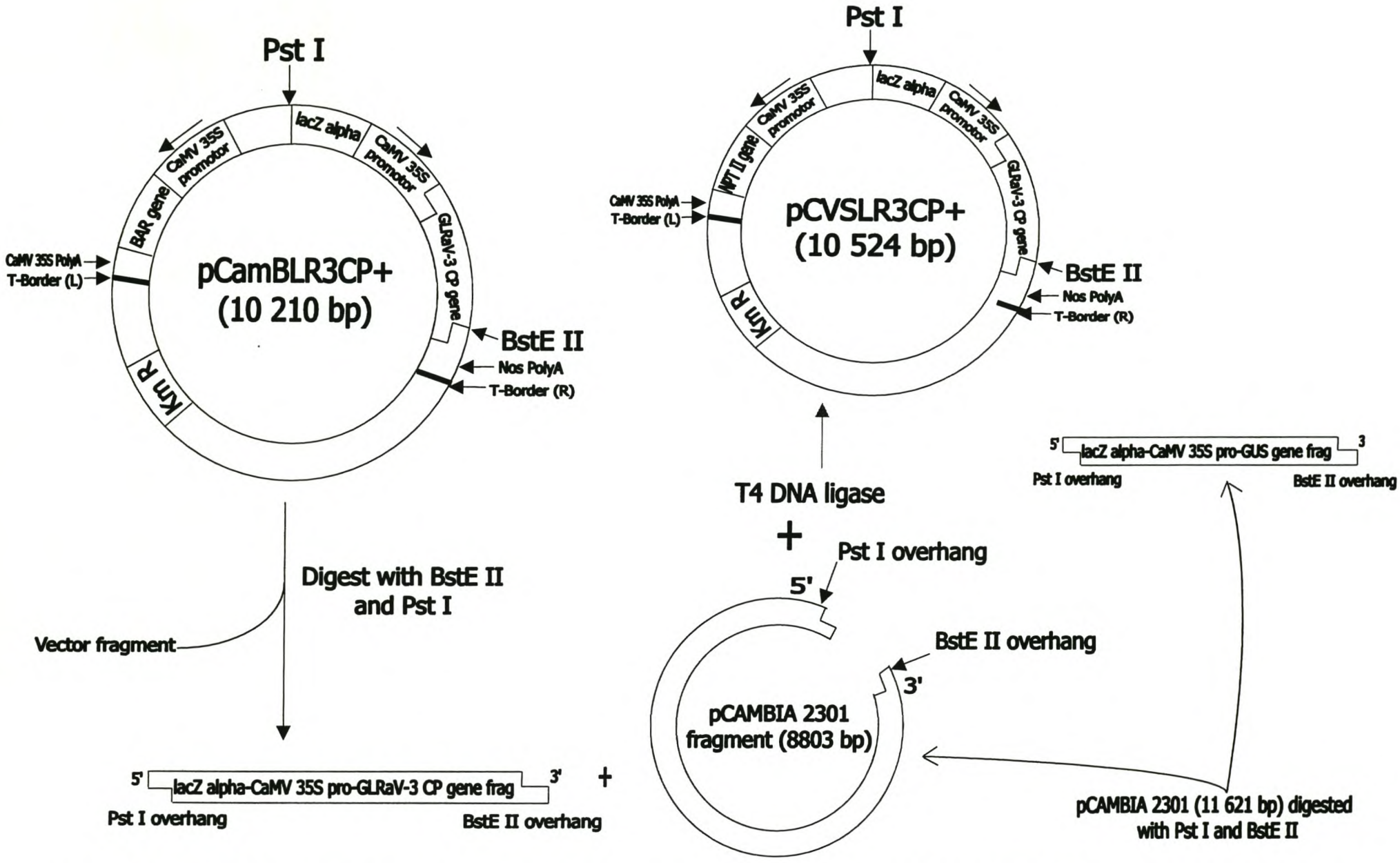

Fig. 3.3. Diagrammatic representation of subcloning the GLRaV-3 CP gene in the sense orientation into pCAMBIA 2301.

from the gel, purified and the concentration determined. Simultaneously, pCAMBIA 2301 was digested with *BstE* II and *Pst* I and electrophoresed to separate the fragments. The 8803 bp fragment corresponding to the pCAMBIA 2301 vector without the lac Z α-CaMV 35S promotor-GUS reporter gene was cut from the gel, purified and the concentration determined. The 1721 bp and 8803 bp fragments were ligated in 30:1, 20:1, 10:1 and 3:1, insert to vector ratios. Colonies were selected for the insert on LB medium containing Km^{100}. Plasmid DNA purifications were performed on overnight bacterial cultures as before. PCR and size analysis was used to confirm the presence of the GLRaV-3 CP gene. The recombinant pCAMBIA 2301 hosting the GLRaV-3 CP gene in the sense orientation was designated as pCVSLR3CP+. Glycerol stocks were made of pCVSLR3CP+ and stored at –80 ºC.

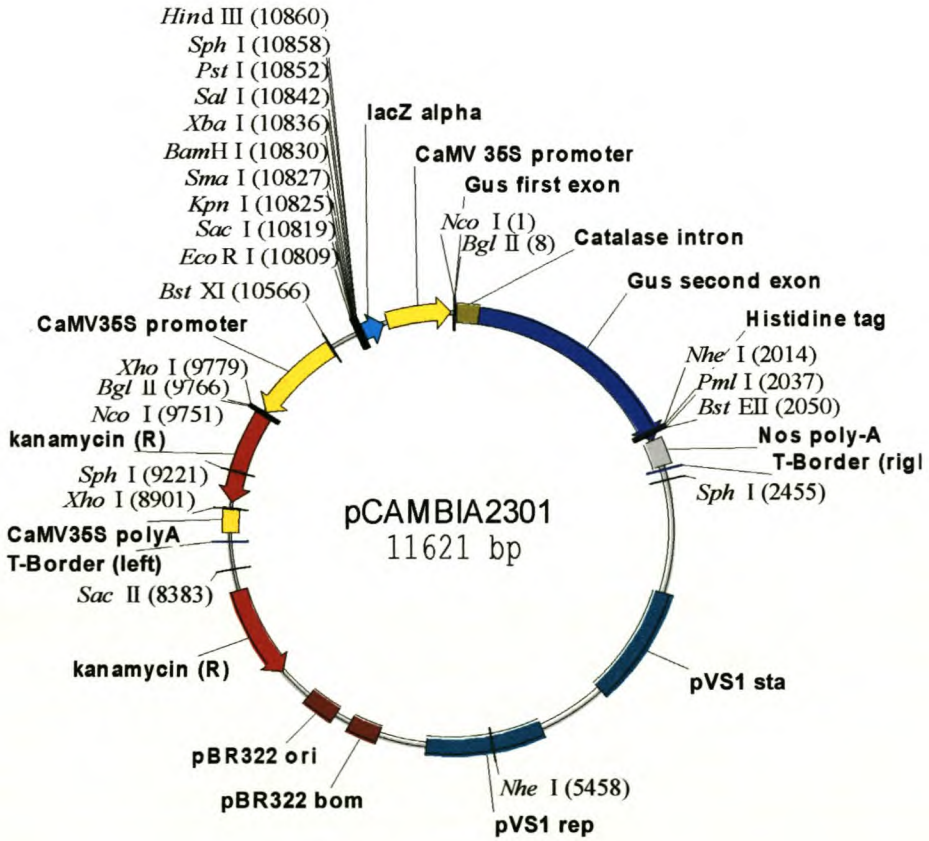

Fig. 3.4. Physical map of pCAMBIA 2301 (courtesy of CAMBIA, Australia).

3.2.3 *Subcloning the GLRaV-3 CP gene in the antisense orientation into pCAMBIA 3301*

Refer to Fig. 3.5 for a diagrammatic representation of the cloning strategy. The GLRaV-3 CP gene was excised from the antisense construct pLR3CP- by digestion with *Apa* I and *Sal* I. The digests were electrophoresed to separate the fragments, and the 1053 bp fragment corresponding to the GLRaV-3 CP gene and flanking RE site nts was cut from a gel, purified and the concentration determined. Simultaneously the vector, pMECA (2860 bp) (Thompson & Parrot, 1998) (Fig. 3.6) was digested with *Apa* I and *Sal* I, removing a small 29 bp fragment. The 2831 bp vector fragment was cut from a gel, purified and the concentration determined. The 1053 bp GLRaV-3 CP gene and 2831 bp pMECA vector fragments were ligated in 5:1, 3:1 and 1:1, insert to vector ratios and transformed into *E. coli* strain DH5α. The putative transformed cells were streaked out on LB medium containing Amp^{100}. Plasmid DNA purifications were performed on overnight bacterial cultures as before. Once again, PCR and size analysis was used to confirm the presence of the GLRaV-3 CP gene. The recombinant pMECA hosting the GLRaV-3 CP gene in the antisense orientation was designated as pMLR3CP- (3884 bp). pMLR3CP- was digested with *Bgl* II and *Sma* I and electrophoresed to separate the fragments. The 1087 bp fragment corresponding to the GLRaV-3 CP gene and flanking RE nts was cut from a gel, purified and the concentration determined. Simultaneously, pCAMBIA 3301 was digested with *Bgl* II and *Pml* I and electrophoresed to separate the fragments. The 9278 bp fragment corresponding to the pCAMBIA 3301 vector without the GUS reporter gene was cut from a gel, purified and the concentration determined. The 1087 bp and 9278 bp fragments were ligated in 30:1, 20:1, 10:1 and 3:1, insert to vector ratios and transformed into *E. coli* strain DH5α. Colonies were selected for the insert on LB medium containing Km^{100}. Plasmid DNA purifications were performed on overnight bacterial cultures as before. Once again, PCR and size analysis was used to confirm the presence of the GLRaV-3 CP gene. The recombinant pCAMBIA 3301 hosting the GLRaV-3

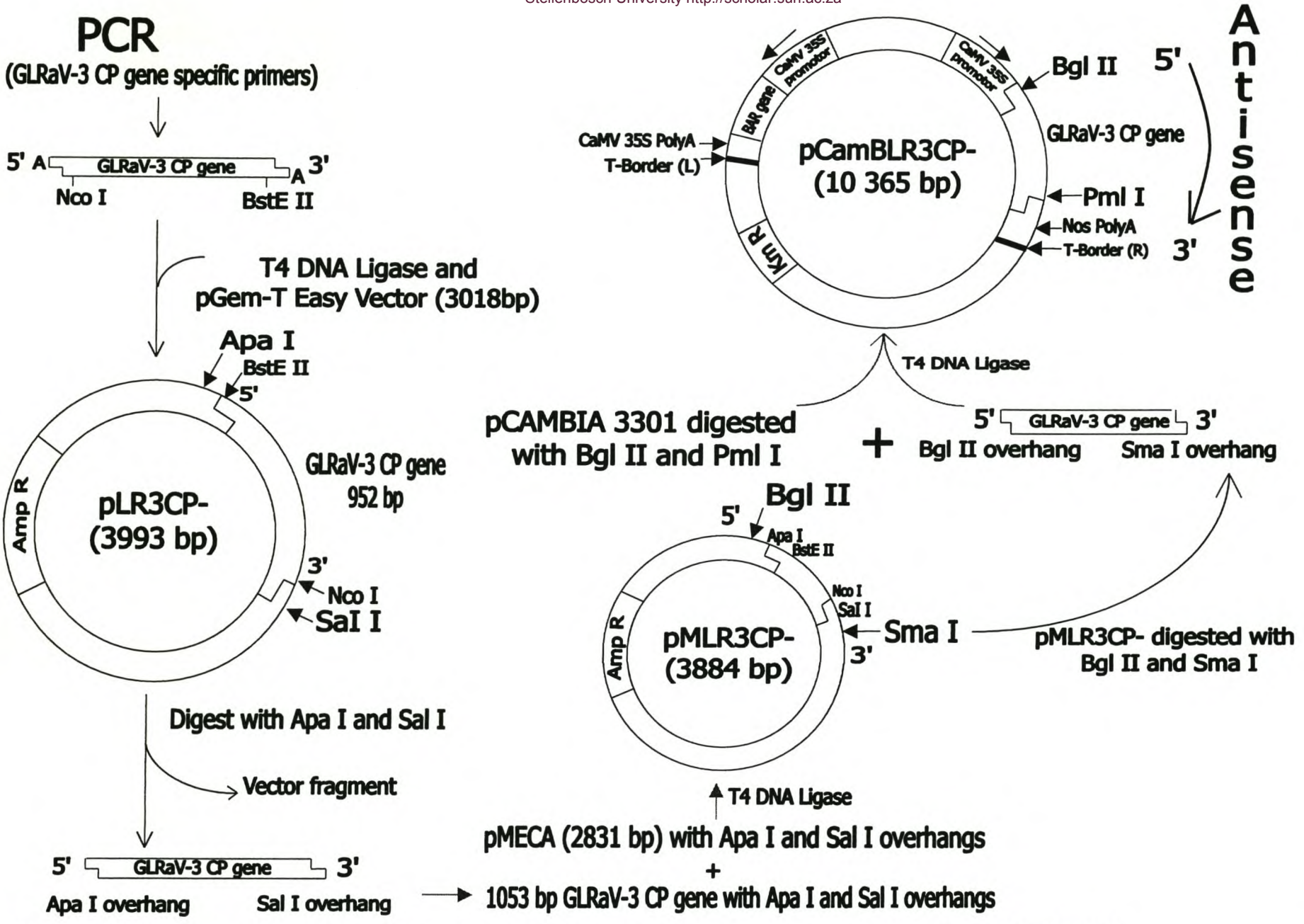

Fig. 3.5. Diagrammatic representation of subcloning the GLRaV-3 CP gene in the antisense orientation into pCAMBIA 3301.

CP gene in the antisense orientation was designated as pCamBLR3CP-. Glycerol stocks were made of pCamBLR3CP- and stored at –80 ºC.

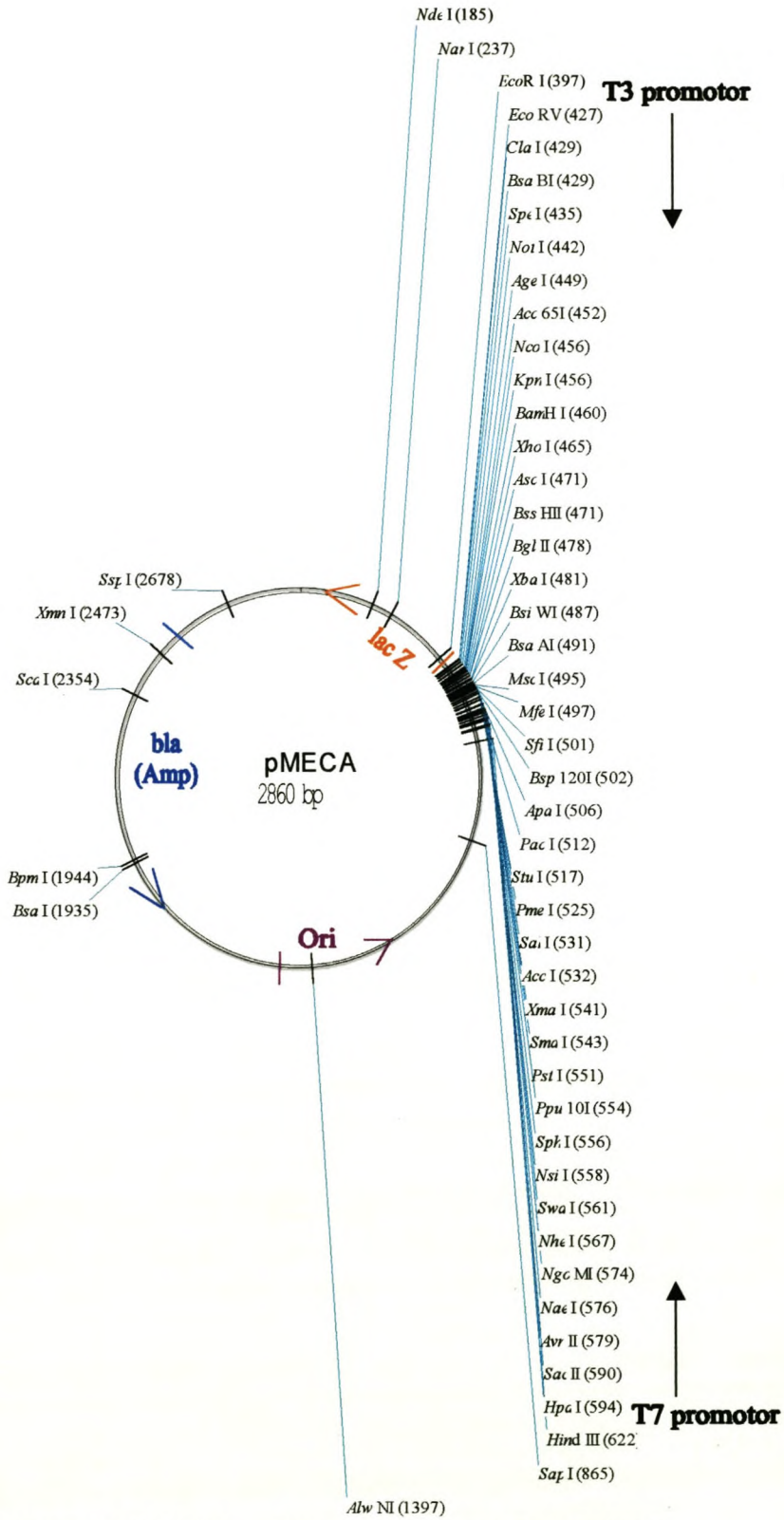

Fig. 3.6. Physical map of pMECA (courtesy of Thomson & Parrot, 1998).

3.3 RESULTS AND DISCUSSION

3.3.1 *Subcloning the GLRaV-3 CP gene in the sense orientation into pCAMBIA 3301*

The sense construct, pLR3CP+ digested with *Nco* I and *BstE* II yielded three fragments. These are a 33 bp fragment, a 952 bp fragment (GLRaV-3 CP gene) containing a *Nco* I site overhang on the 5' end and a *BstE* II site overhang on the 3' end and a 3008 bp fragment corresponding to the remaining pLR3CP+ vector. As depicted in Fig. 3.7, the 33 bp fragment could not be seen on the 1.4 % TAE agarose gel, but the 952 bp GLRaV-3 CP gene fragment could clearly be identified and gel purified.

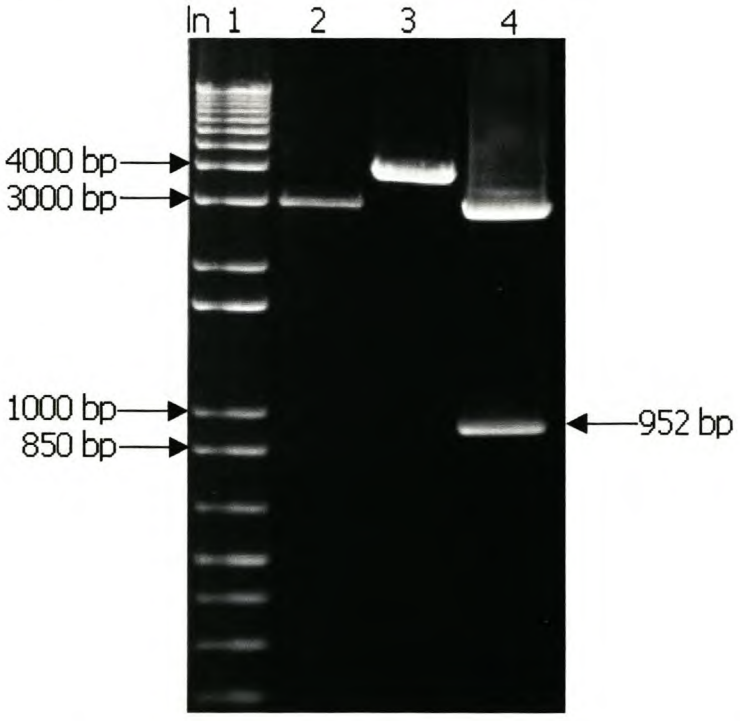

Fig. 3.7. Confirmation of the 952 bp GLRaV-3 CP gene fragment liberated from pLR3CP+. Lane 1, 1 kb plus DNA ladder. Lane 2, linear pGem®-T Easy vector (3018 bp). Lane 3, pGem®-T Easy vector with the GLRaV-3 CP gene insert (pLR3CP+) (3993 bp) linearized with *Pst* I. Lane 4, pLR3CP+ digested with *Nco* I and *BstE* II produces three fragments, two of which can be seen on the gel. These are the 3008 bp pLR3CP+ vector fragment and the 952 bp GLRaV-3 CP gene fragment.

Digestion of pCAMBIA 3301 with the same two REs produced two fragments, the 2049 bp GUS reporter gene and the 9258 bp fragment corresponding to the remaining pCAMBIA 3301 vector (Fig. 3.8). The latter fragment has a *Nco* I site overhang just downstream of the CaMV 35S promotor and a *BstE* II site overhang just upstream of the NOS poly A terminator (refer to Fig. 3.2).

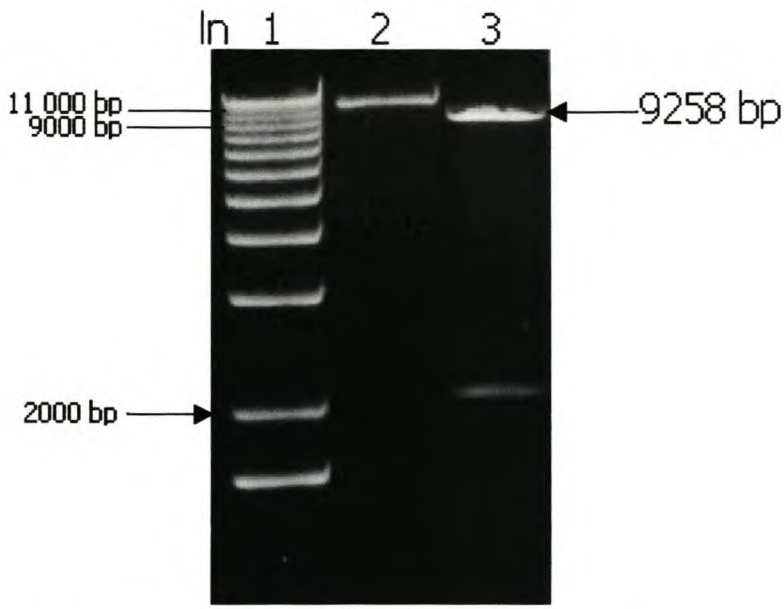

Fig. 3.8. Confirmation of the 9258 bp pCAMBIA 3301 vector fragment subsequent to removing the 2049 bp GUS reporter gene. Lane 1, 1 kb plus DNA ladder. Lane 2, pCAMBIA 3301 linearized with *Pst* I (11 307 bp). Lane 3, pCAMBIA 3301 digested with *Nco* I and *BstE* II produces two fragments. These are the 2049 bp GUS reporter gene and the 9258 bp pCAMBIA vector fragment, both of which can be seen clearly on the gel.

Ligation of the 9258 bp pCAMBIA vector fragment and the 952 bp GLRaV-3 CP gene fragment only worked when a 30:1, insert to vector ratio and no more than 2 µl of the ligation reaction mix was used. Only three putatively transformed colonies were present on the LB medium and Km^{100} plates. PCR (results not shown) and size analysis confirmed that the three colonies contained the recombinant pCAMBIA 3301 hosting the GLRaV-3 CP gene (pCamBLR3CP+). As depicted in Fig. 3.9, size analysis on a 0.8 % TAE

agarose gel shows that pCamBLR3CP+ is 10 210 bp in size, which is as a result of the 2049 bp (GUS reporter gene) loss and the 952 bp (GLRaV-3 CP gene fragment) gain.

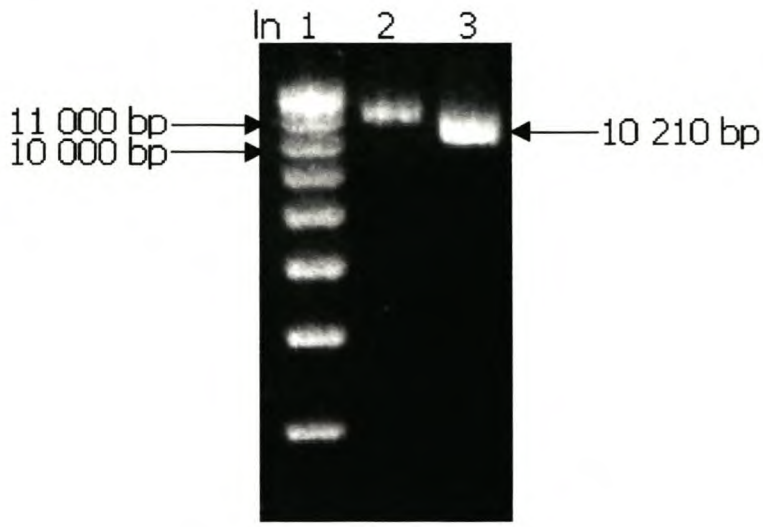

Fig. 3.9. Size analysis of pCamBLR3CP+ on a 0.8 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lane 2, pCAMBIA 3301 linearized with *Pst* I (11 307 bp). Lane 3, pCamBLR3CP+ linearized with *Pst* I is 10 210 bp in size, which is as a result of the 2049 bp (GUS reporter gene) loss and the 952 bp (GLRaV-3 CP gene fragment) gain.

3.3.2 *Subcloning the GLRaV-3 CP gene in the sense orientation into pCAMBIA 2301*

Digestion of pCamBLR3CP+ with *BstE* II and *Pst* I cuts the construct into two fragments, the 1721 bp fragment, which corresponds to the lac Z α-CaMV 35S promotor-GLRaV-3 CP gene and a 8489 bp fragment corresponding to the remainder of pCamBLR3CP+. The 1721 bp fragment could clearly be identified and gel purified (Fig. 3.10). Digestion of pCAMBIA 2301 with the same two REs cuts the vector into two fragments, the lac Z α-CaMV 35S promotor-GUS reporter gene fragment (2818 bp) and the remainder of pCAMBIA 2301, a fragment of size 8803 bp (Fig. 3.11). The ligation of this 8803 bp pCAMBIA 2301 vector fragment and the 1721 bp lac Z α-CaMV 35S promotor-GLRaV-3 CP gene fragment was successful when a 10:1, insert to vector ratio was used. PCR (results not shown) and size analysis confirmed

that the colonies screened contained the recombinant pCAMBIA 2301 hosting the GLRaV-3 CP gene (pCVSLR3CP+). As depicted in Fig. 3.11, size analysis shows that pCVSLR3CP+ is 10 524 bp in size, which is as a result of the 2818 bp (lac Z α-CaMV 35S promotor-GUS reporter gene fragment) loss and the 1721 bp (lac Z α-CaMV 35S promotor-GLRaV-3 CP gene fragment) gain.

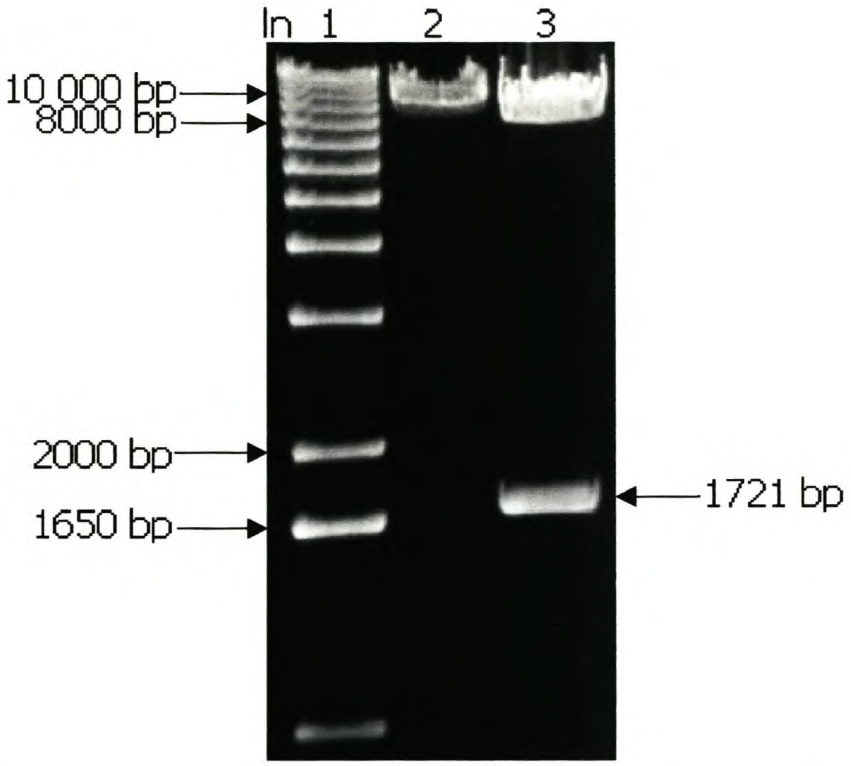

Fig. 3.10. Confirmation of the 1721 bp lac Z α-CaMV 35S promotor-GLRaV-3 CP gene fragment liberated from pCamBLR3CP+. Lane 1, 1 kb plus DNA ladder. Lane 2, pCamBLR3CP+ linearized with *Pst* I (10 210 bp). Lane 3, pCamBLR3CP+ digested with *BstE* II and *Pst* I produces two fragments. These are the 1721 bp lac Z α-CaMV 35S promotor-GLRaV-3 CP gene fragment and the remaining 8489 bp pCamBLR3CP+ vector fragment, both of which can be seen clearly on the gel.

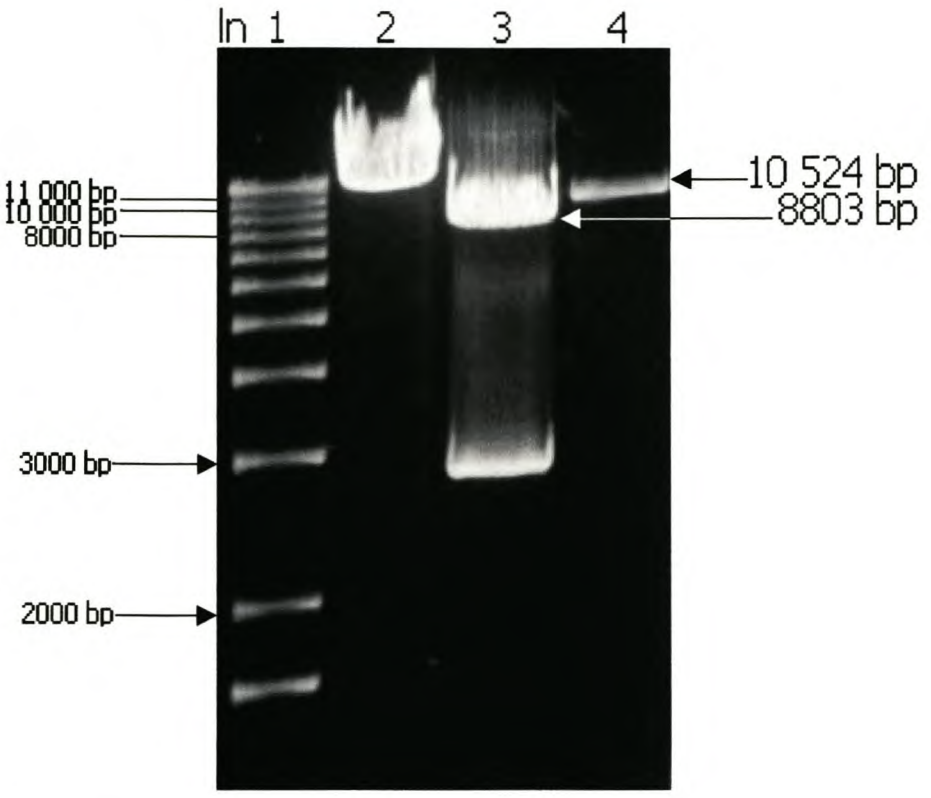

Fig. 3.11. Confirmation of the 8803 bp pCAMBIA 2301 vector fragment subsequent to removing the 2818 bp lac Z α-CaMV 35S promotor-GUS reporter gene fragment and size analysis of pCVSLR3CP+. Lane1, 1 kb plus DNA ladder. Lane 2, pCAMBIA 2301 linearized with *Pst* I (11 621 bp). Lane 3, pCAMBIA 2301 digested with *BstE* II and *Pst* I produces two fragments. These are the 2818 bp lac Z α-CaMV 35S promotor-GUS reporter gene fragment and the remaining 8803 bp vector fragment, both of which can be seen clearly on the gel. Lane 4, the recombinant pCAMBIA 2301 hosting the GLRaV-3 CP gene (pCVSLR3CP+) linearized with *Pst* I is 10 524 bp in size.

3.3.3 *Subcloning the GLRaV-3 CP gene in the antisense orientation into pCAMBIA 3301*

As mentioned before, the construct pLR3CP- hosts the GLRaV-3 CP gene in the antisense orientation as determined by RE analysis. The small plasmid, pMECA was used as an intermediate vector for subcloning the GLRaV-3 CP gene from pLR3CP- into pCAMBIA 3301, thus maintaining the antisense orientation of the GLRaV-3 CP gene at all times during the subcloning steps. This vector was chosen because of the presence of useful RE sites to facilitate the subcloning of the GLRaV-3 CP gene in the antisense orientation.

Digestion of pLR3CP- with *Apa* I and *Sal* I produces two fragments, a 1053 bp fragment corresponding to the GLRaV-3 CP gene with flanking RE site nts and the remaining pLR3CP- vector fragment of size 2940 bp (Fig. 3.12).

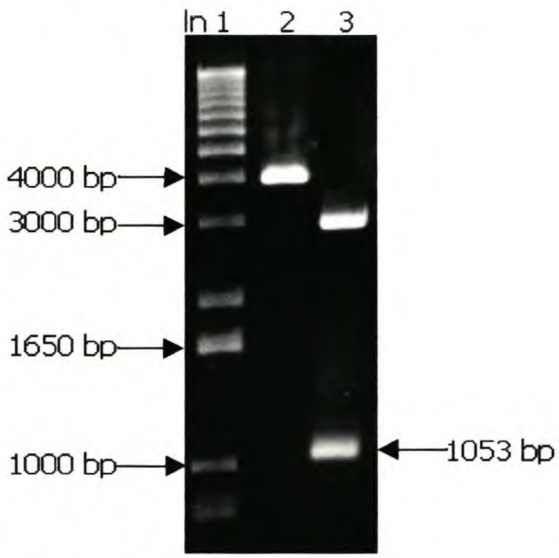

Fig. 3.12. Confirmation of the 1053 bp GLRaV-3 CP gene fragment liberated from pLR3CP-. Lane 1, 1 kb plus DNA ladder. Lane 2, pLR3CP- linearized with *Pst* I (3993 bp). Lane 3, pLR3CP- digested with *Apa* I and *Sal* I produces two fragments. These are the 2940 bp vector fragment and the 1053 bp GLRaV-3 CP gene fragment, both of which can be seen clearly on the gel.

Digestion of pMECA with *Apa* I and *Sal* I liberates a small 29 bp fragment. It can only be assumed that the digestion was complete, as the small difference in size between digested and undigested pMECA can not be seen on the 1.4 % TAE agarose gel (Fig. 3.13). The resultant 2831 bp pMECA vector fragment with *Apa* I and *Sal* I site overhangs and the 1053 bp GLRaV-3 CP gene fragment were successfully ligated in a 3:1, insert to vector ratio. PCR (results not shown) and size analysis confirmed that the colonies screened contained the recombinant pMECA hosting the GLRaV-3 CP gene (pMLR3CP-). As is also depicted in Fig. 3.13, size analysis shows that pMLR3CP- is 3884 bp in size, which is as a result of the 29 bp loss and the 1053 bp (GLRaV-3 CP gene fragment) gain. Digestion of pMLR3CP- with *Bgl* II and *Sma* I, once again produced two fragments, the 1087 bp GLRaV-3 CP gene fragment (maintaining the antisense orientation) and the remainder of pMLR3CP- (2797 bp). Digestion of pCAMBIA 3301 with *Bgl* II and *Pml* I

produced two fragments, the 2029 bp GUS reporter gene and the remaining 9278 bp pCAMBIA 3301 vector fragment (Fig. 3.14). The latter fragment has a *Bgl* II site overhang just downstream of the CaMV 35S promotor and a *Pml* I site overhang just upstream of the NOS poly A terminator (refer to Fig. 3.2). Ligation of this 9278 bp pCAMBIA 3301 vector fragment and the 1087 bp GLRaV-3 CP gene fragment with *Bgl* II and *Sma* I overhangs (*Sma* I and *Pml* I are blunt cutters) was successful when a 20:1, insert to vector ratio was used. PCR (results not shown) and size analysis confirmed that the colonies screened contained the recombinant pCAMBIA 3301 hosting the GLRaV-3 CP gene (pCamBLR3CP-). As depicted in Fig. 3.15, size analysis shows that pCamBLR3CP- is 10 365 bp in size, which is as a result of the 2029 bp (GUS reporter gene) loss and the 1087 bp (GLRaV-3 CP gene fragment) gain.

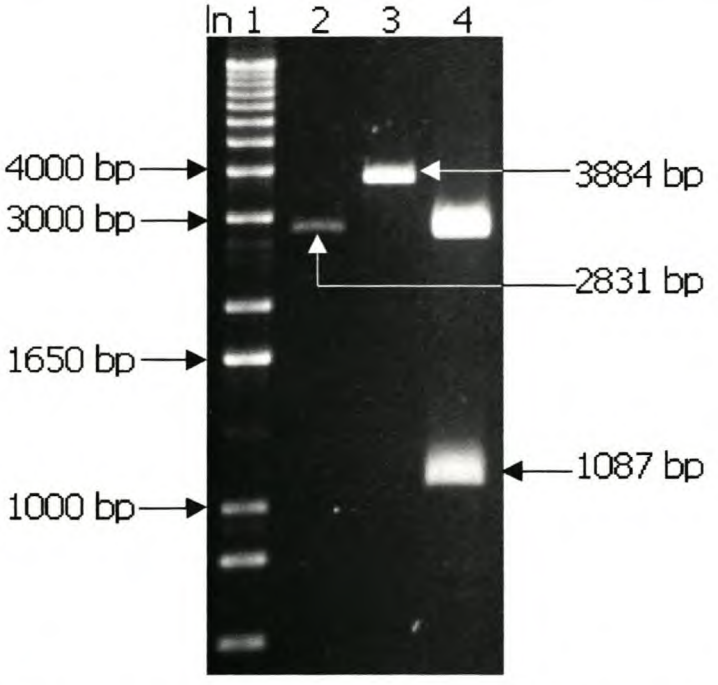

Fig. 3.13. Confirmation of the 2831 bp pMECA vector fragment, size analysis of pMLR3CP- and confirmation of the 1087 bp GLRaV-3 CP gene fragment. Lane 1, 1 kb plus DNA ladder. Lane 2, pMECA digested with *Apa* I and *Sal* I produces two fragments, the 2831 bp vector fragment (as can be seen on the gel) for use in a ligation reaction and the small 29 bp fragment which can not be seen on the gel. Lane 3, the recombinant vector, pMLR3CP- linearized with *Pst* I (3884 bp). Lane 4, pMLR3CP- digested with *Sma* I and *Bgl* II produces two fragments. These are the 1087 bp GLRaV-3 CP gene fragment for use in a ligation reaction and the remaining vector fragment (2797 bp), both of which can be seen clearly on the gel.

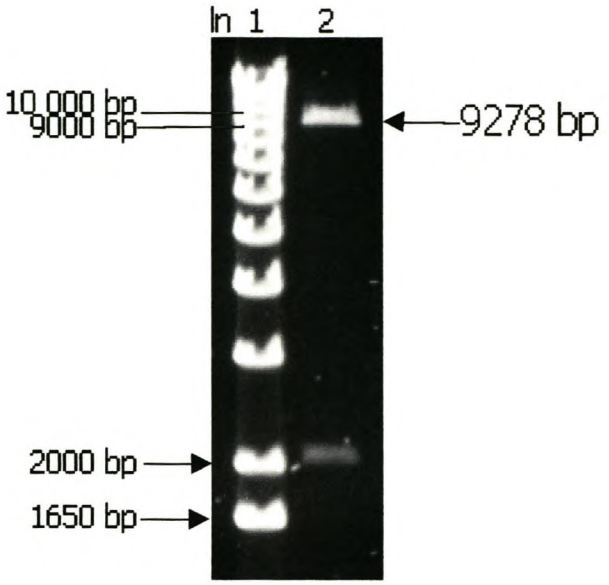

Fig. 3.14. Confirmation of the 9278 bp pCAMBIA 3301 vector fragment subsequent to removing the 2029 bp GUS reporter gene. Lane 1, 1 kb plus DNA ladder. Lane 2, pCAMBIA 3301 digested with *Bgl* II and *Pml* I produces two fragments. These are the 2029 bp GUS reporter gene and the remaining 9278 bp pCAMBIA 3301 vector fragment, both of which can be seen clearly on the gel.

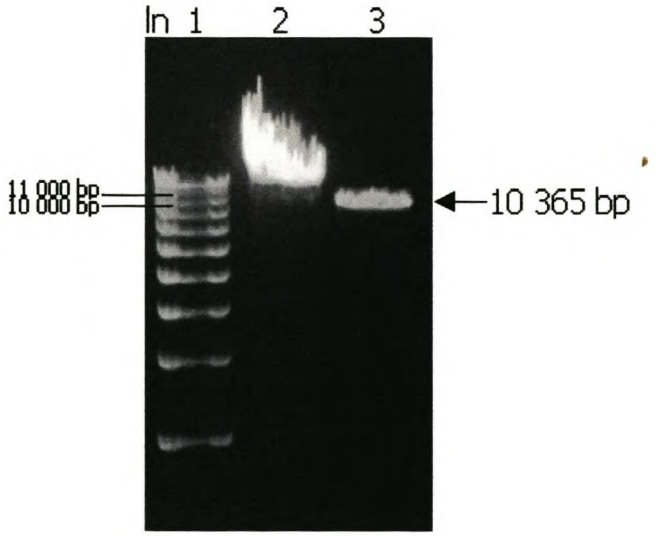

Fig. 3.15. Size analysis of pCamBLR3CP- on a 1.4 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lane 2, pCAMBIA 3301 linearized with *Pst* I (11 307 bp). Lane 3, pCamBLR3CP- linearized with *Pst* I is 10 365 bp in size, which is as a result of the 2029 bp (GUS reporter gene) loss and the 1087 bp (GLRaV-3 CP gene fragment) gain.

3.4 CONCLUDING REMARKS

The GLRaV-3 CP gene was excised from pLR3CP+ and pLR3CP-, which host the GLRaV-3 CP gene in the sense and antisense orientations respectively. The CP gene was subcloned into the plant expression vector, pCAMBIA 3301 in the sense and antisense orientations. The CP gene was also subcloned into another plant expresssion vector, pCAMBIA 2301 in the sense orientation. Each of these vectors contains a multiple cloning site and two CaMV 35S promotors, one driving the expression of the GUS reporter gene and one driving the expression of either the Km resistance gene in the case of pCAMBIA 2301, or the *bar* herbicide resistance gene in the case of pCAMBIA 3301. This is all situated within the region between the right and left borders, and is therefore also the region that is transferred via *A. tumefaciens* to the tobacco plant cell genome (Wang *et al.*, 1984). It was decided to remove the GUS reporter gene in all these cloning strategies and replace it with the CP gene. The CP gene would therefore be subcloned downstream of a CaMV 35S promotor for subsequent expression. The main reason for not cloning into the multiple cloning sites, was the need to then introduce a third promotor to drive the expression of the CP gene. A CaMV 35S promotor would have very likely been used, since it is 30-50 times stronger than the NOS promotor (Sanders *et al.*, 1987). The presence of three CaMV 35S promotors could very likely result in gene silencing (Wassenegger & Pélissier, 1998). Gene silencing is not very well understood, but it is characterized by a reduced gene expression caused by a high copy number of a particular sequence. Multiple copies of the same gene can therefore result in sequence-specific suppression of RNA accumulation and methylation of the corresponding gene.

These three recombinant constructs were given to M. Vivier for use in grapevine transformation experiments. Since pCAMBIA 3301 and pCAMBIA 2301 contains the *bar* and Km resistance genes respectively, the selective agents, Basta and Km will be evaluated for their effects on grapevine material. In conclusion, the GLRaV-3 CP gene was subcloned into plant expression vectors for tobacco and grapevine transformation experiments.

CHAPTER 4

AGROBACTERIUM -MEDIATED TRANSFORMATION OF TOBACCO WITH THE RECOMBINANT BINARY VECTORS, pCamBLR3CP+ and pCamBLR3CP-

SUMMARY

The recombinant plant expression vectors, pCamBLR3CP+ and pCamBLR3CP- were designed to host the GLRaV-3 CP gene for sense and antisense expression respectively, in transgenic plants. These two constructs were used to transform *Nicotiana tabacum* L.cv.'Xanthi' leaf discs by *Agrobacterium tumefaciens*-mediated transformation. Included as a positive control were leaf discs transformed by *A. tumefaciens* harboring pCAMBIA 3301. Leaf discs transformed only with *A. tumefaciens* were used as a negative control. Shoots were regenerated on plant tissue culture medium containing the selective agent, Basta. The ability to withstand this herbicide is due to the presence of a plant selectable marker gene on each of these constructs, known as the *bar* gene. Tobacco plants putatively transformed with pCamBLR3CP+, pCamBLR3CP- and pCAMBIA 3301 survived on medium containing Basta, whereas the negative control plants died.

4.1 INTRODUCTION

The soil-borne bacterium, *A. tumefaciens* causes crown gall disease in most dicotyledonous plants by genetically transforming plant cells with a specific DNA region, the T-DNA, from its tumour inducing (Ti) plasmid to the plant cell nucleus (Cado, 1991). The virulence (*vir*) genes, which are situated elsewhere on the Ti plasmid, are transcriptionally activated in response to a chemical secreted by wounded plant cells, called acetosyringone (Stachel *et al.*, 1985). These genes are essential for the transfer of the T-DNA region to the plant cell nucleus (Stachel & Zambryski, 1986). The T-DNA is flanked by 25 bp sequence repeats known as the left and right borders. One or both of these are required for the excision and transfer of the T-DNA region (Wang *et al.*, 1984). The T-DNA region has been genetically modified to act as a vector for transferring genes of interest to the genomes of plant cells via *A. tumefaciens*. Tobacco transformation and subsequent regeneration can be performed with relative ease using the leaf disc technique. Tobacco has become a model plant for research on the control of plant gene expression by looking at modified or chimeric transgenes. The *A. tumefaciens* binary vector system for transforming plant cells is based on the fact that only the *cis*-acting elements are required for T-DNA transfer and that the T-DNA and the *vir* genes can be carried on separate plasmids (Hoekema *et al.*, 1983). This binary vector system requires the following for transformation: a binary plant expression vector, which carries the T-DNA region (refer to par. 3.1) and a *vir* plasmid, which is a modified Ti plasmid that lacks the T-DNA region but contains the *vir* genes required for T-DNA transfer. A "helper plasmid" is also required for conjugation between the *E. coli* hosting the binary vector and the *A. tumefaciens* hosting the *vir* plasmid. This conjugative mating is referred to as a triparental mating. The "helper" plasmid induces the conjugative transfer of the binary vector from *E. coli* to *A. tumefaciens* (reviewed in Watson *et al.*, 1992). The transformation of tobacco plant cells is achieved by incubating the leaf discs with the *A. tumefaciens* carrying the binary vector (Horsch *et al.*, 1985).

This chapter reports the *Agrobacterium*-mediated transformation of tobacco plants by using the binary vector system. The recombinant binary plant expression vectors, pCamBLR3CP+ and pCamBLR3CP-, which host the CP gene of GLRaV-3 in the sense and antisense orientations respectively, were used for the tobacco transformation. This is done to test for transgene expression since GLRaV-3 can not be transmitted to tobacco. The transformation and regeneration of the tobacco leaf discs are discussed.

4.2 MATERIALS AND METHODS

4.2.1 *Triparental matings*

Single colonies of *E. coli* strain DH5α harboring pCamBLR3CP+ (donor), pCamBLR3CP- (donor), pCAMBIA 3301 (donor) and pRK 2013 ("helper") separately were grown overnight at 37 ºC in LB medium and Km^{100}. Simultaneously, the avirulent *A. tumefaciens* strain LBA 4404 was grown at 28 ºC in LB medium containing rifampicin at a concentration of 100 µg/ml (Rif^{100}) for 36 hours. The optical density (OD) of each sample was read at 600 nm on a spectrophotometer. Triparental matings were performed by pipetting 100 µl of each culture (donor, *A. tumefaciens* and helper pRK 2013) on solidified LB medium without selective antibiotics and incubated for 36 hours at 28 ºC (Table 4.1). A scrape of the resultant bacterial lawn was streaked on LB medium containing Km^{100} and Rif^{100} and incubated for a further 36 hours at 28 ºC. Putatively recombinant *A. tumefaciens* colonies were thus selected on LB medium containing Km^{100} and Rif^{100}. The putative recombinant *A. tumefaciens* colonies harboring either pCamBLR3CP+ or pCamBLR3CP- were screened for the presence of the GLRaV-3 CP gene by PCR. The putative recombinant *A. tumefaciens* colonies harboring pCAMBIA 3301 were screened for the presence of the *bar* gene by PCR. *Bar* gene specific primers were obtained from J.T. Burger. These positive colonies were used to inoculate LB medium containing selective antibiotics as described in Table 4.2 for subsequent infection of tobacco leaf discs.

Table 4.1. Representation of the three triparental matings performed. Single colonies of each donor, pCamBLR3CP+ (no.1) and pCamBLR3CP- (no.2) were transferred to *A. tumefaciens* recipients using the conjugative "helper plasmid", pRK 2013. A third triparental mating using pCAMBIA 3301 as donor was included as a positive control (no.3).

1. Triparental mating 1	*2. Triparental mating 2*	*3. Triparental mating 3*
100 µl pCamBLR3CP+ (sense)	100 µl pCamBLR3CP- (antisense)	100 µl pCAMBIA 3301 (positive control)
+	+	+
100 µl Agro LBA 4404	100 µl Agro LBA 4404	100 µl Agro LBA 4404
+	+	+
100 µl pRK 2013	100 µl pRK 2013	100 µl pRK 2013

4.2.2 *Plant transformation and regeneration*

Young *Nicotiana tabacum* L.cv.'Xanthi' plants were kindly donated by B. Von Wechmar. Leaves were surface sterilized in a 0.5 % calcium hypochorite solution for 3 minutes and rinsed thoroughly 4x with autoclaved H_2O. Two hundred leaf explants (~1.0 cm^2) were cut under sterile conditions and placed on Murashige and Skoog (MS) plant tissue culture medium (Murashige and Skoog, 1962) prepared according to the manufacturer's instructions (Sigma-Aldrich). The leaf discs were incubated at 24 °C in low light intensity incubators and after 2 days the explants were dipped in recombinant *A. tumefaciens* cultures as described in Table 4.2. The leaf discs were placed back on MS medium and incubated in the dark at 24 °C for 4 days until *A. tumefaciens* growth appeared.

Table 4.2. Representation of the four *A. tumefaciens* cultures prepared for infection of leaf discs. The positive recombinant *A. tumefaciens* colonies harboring either pCamBLR3CP+ (no.1), pCamBLR3CP- (no.2) or pCAMBIA 3301 (no.3) were used to inoculate LB medium containing Km^{100} and Rif^{100}. One non-recombinant *A. tumefaciens* colony was used to inoculate LB medium containing only Rif^{100} for use as a negative control (no.4).

1.LB+Rif^{100}+Km^{100} inoc with LBA 4404:pCamBLR3CP+ (triparental mating no.1) .....70 leaf discs
2. LB+Rif^{100}+Km^{100} inoc with LBA 4404:pCamBLR3CP- (triparental mating no. 2) .....70 leaf discs
3. LB+Rif^{100}+Km^{100} inoc with LBA 4404:pCAMBIA 3301 (triparental mating no. 3) .....30 leaf discs
4. LB+Rif^{100} inoc with LBA 4404 (negative control) .....30 leaf discs

Leaf discs were then transferred to fresh MS medium containing the plant growth hormones, 1-napthaleneacetic acid (1-NAA) (Sigma-Aldrich) at a concentration of 0.1 mg/l and 6-benzylaminopurine (6-BAP) (Sigma-Aldrich) at a concentration of 1.0 mg/l. A broad-spectrum antibiotic, cefotaxime (Cx) (Claforan®, Roussel), was also added in the medium at a concentration of 500 µg/ml to kill off the *A. tumefaciens*. Once the *A. tumefaciens* was totally removed the Cx was omitted from the medium. Leaf discs were incubated at 24 °C at a low light intensity until shoots appeared and then placed on medium containing the selective agent, Basta (Agrevo), which was added at a concentration of 0.0025 mg/ml. After 3 weeks, 6-BAP was omitted from the medium to induce root growth. After 2 months, the young plants were hardened off and planted in autoclaved soil. Plants were protected by plastic bag coverings and fed a mixture of liquid MS medium and autoclaved H_2O and kept under a light-dark regime (16 hours of light at 24 °C and 8 hours of dark at 20 °C). After 6 weeks there was enough material for further analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 *Triparental matings*

Single colonies of *E. coli* were verified to contain the plant expression vectors pCamBLR3CP+, pCamBLR3CP- and pCAMBIA 3301 by PCR with the GLRaV-3 CP gene specific and *bar* gene specific primers respectively (results not shown). *E. coli* colonies containing pRK 2013 were donated by M.J. Freeborough. These verified colonies were used to inoculate LB medium and Km^{100}. These bacterial cultures reached an $OD_{600\ nm}$ of ~1 after a 14 hour growth period at 37 ºC. A single colony of *A. tumefaciens* strain LBA 4404 was used to inoculate LB medium and Rif^{100} and reached an $OD_{600\ nm}$ of ~1 only after 36 hours of growing at 28 ºC. These 3 bacterial suspensions were used to perform the triparental matings and after 2 days yielded a number of putative recombinant *A. tumefaciens* colonies. It was assumed that these colonies contained a donor plasmid (pCamBLR3CP+, pCamBLR3CP- or pCAMBIA 3301) because these three donors all have a bacterial Km resistance gene and *A. tumefaciens* has a Rif resistance gene. PCR confirmed that the putative recombinant *A. tumefaciens* colonies harboring either pCamBLR3CP+ or pCamBLR3CP- contained the 975 bp GLRaV-3 CP gene (Fig. 4.1). PCR also confirmed that the putative recombinant *A. tumefaciens* colonies harboring pCAMBIA 3301 contained the ~560 bp *bar* gene (Fig. 4.2). These same positive colonies were used to inoculate LB medium containing Km^{100} and Rif^{100}. These cultures reached an $OD_{600\ nm}$ of ~1 after a 36 hour growth period at 28 ºC. These recombinant *Agrobacterium* suspensions were then used for infecting the leaf discs.

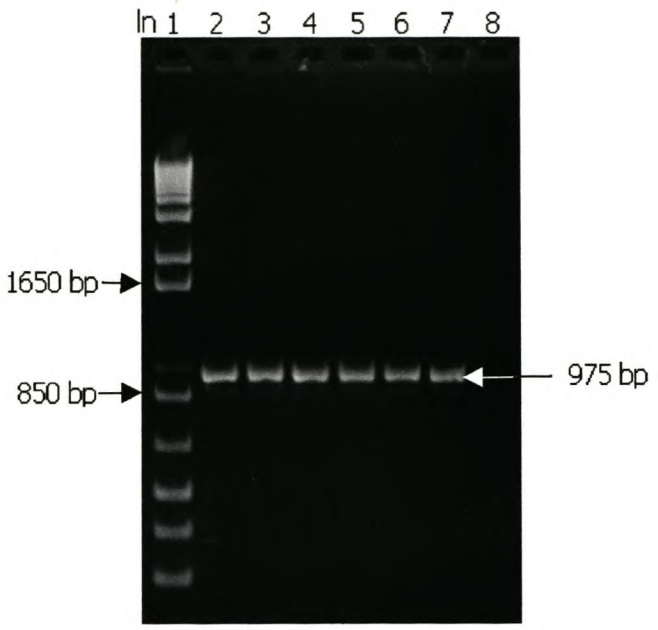

Fig. 4.1. PCR analysis (using GLRaV-3 CP gene specific primers) on putative recombinant *A. tumefaciens* colonies. Lane 1, 1 kb plus DNA ladder. Lane 2-4, amplification of the 975 bp GLRaV-3 CP gene confirmed the presence of pCamBLR3CP+ in the corresponding *A. tumefaciens* colonies. Lane 5-7, the same applied for colonies harboring pCamBLR3CP-. Lane 8, H_2O was included in the PCR reaction as a negative control.

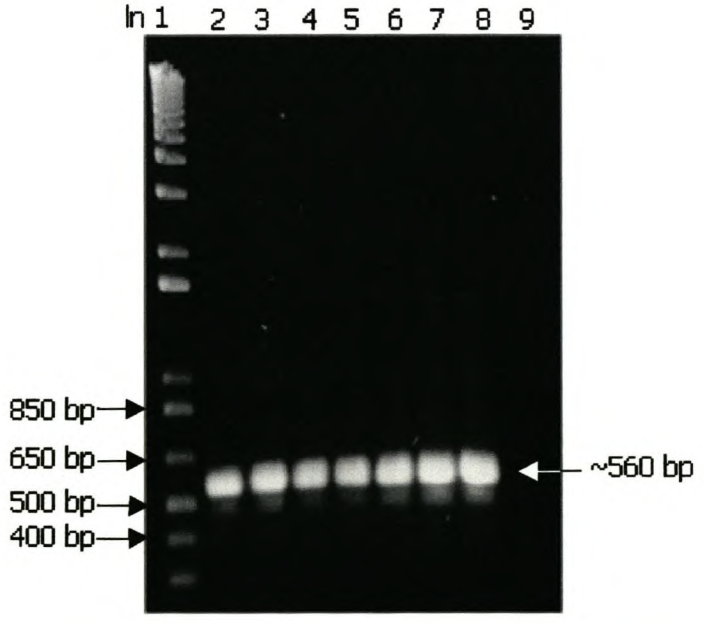

Fig. 4.1. PCR analysis (using *bar* gene specific primers) on putative recombinant *A. tumefaciens* colonies. Lane 1, 1 kb plus DNA ladder. Lane 2-8, amplification of the ~560 bp *bar* gene confirmed the presence of pCAMBIA 3301 in the corresponding *A. tumefaciens* colonies. Lane 9, H_2O was included in the PCR reaction as a negative control.

4.3.2 *Plant transformation and regeneration*

Tobacco (*Nicotiana tabacum* L.cv.'Xanthi') leaf discs were transformed by *Agrobacterium tumefaciens*-mediated transformation. Approximately 70 leaf discs were infected with *A. tumefaciens* carrying pCamBLR3CP+ and ~70 leaf discs with *A. tumefaciens* carrying pCamBLR3CP-. Approximately 30 leaf discs were infected with *A. tumefaciens* carrying pCAMBIA 3301 for regeneration as positive control plants and ~30 leaf discs were infected with *A. tumefaciens* only, for regeneration as negative control plants.
A. tumefaciens growth was clearly visible after 4 days and was effectively killed with Cx at a concentration of 500 µg/ml. After addition of the plant growth hormones, callus formation appeared within 2-3 weeks and after another 2-3 weeks shoots appeared (Fig. 4.3). The shoots were dissected from the leaf discs and placed on MS medium containing the herbicide, Basta. To prevent contamination problems, shoots were transferred to fresh medium only when absolutely necessary and under extreme sterile conditions. The shoots putatively transformed with pCamBLR3CP+, pCamBLR3CP- and pCAMBIA 3301 survived on the MS medium containing Basta and rooted three weeks after the 6-BAP was omitted (Fig. 4.4).

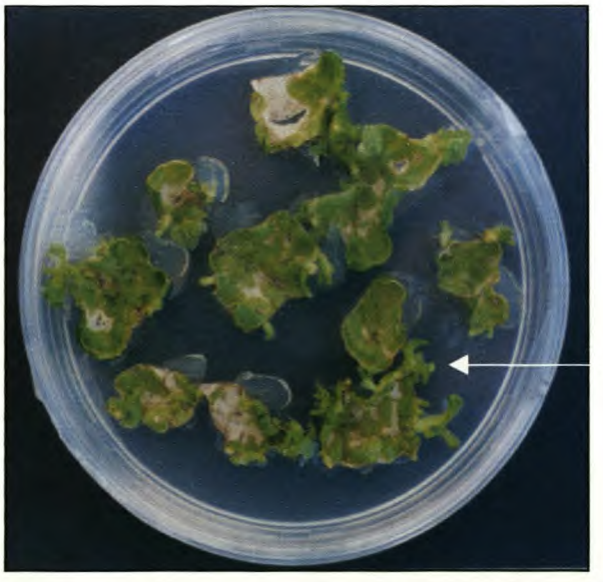

Fig. 4.3. Appearance of "antisense" tobacco leaf discs plated on MS medium containing the plant growth hormones, 1-NAA and 6-BAP. Arrow, shoot development was seen after ~4-6 weeks.

It was expected that "sense", "antisense" and "positive control" shoots would survive on Basta due to the presence of the *bar* gene on each of these plant expression vectors. Also, as expected, the "negative control" shoots did not survive on Basta and died after only a few days (Fig. 4.5). A total of 11 putative transformants (2 "sense", 6 "antisense", and 3 "positive controls") were regenerated on selective medium as healthy tobacco plants that could be hardened off and used for molecular analysis (Fig. 4.6).

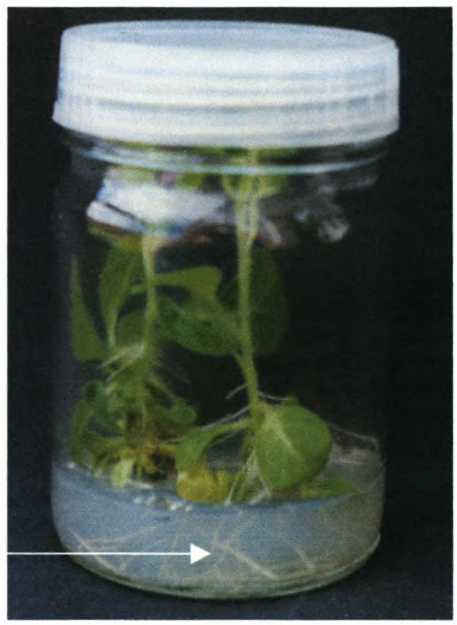

Fig. 4.4. Appearance of young "antisense" tobacco plants on MS medium containing the selective agent, Basta and the plant growth hormone, 1-NAA. Arrow, roots started developing ~3 weeks after the hormone, 6-BAP was omitted from the medium.

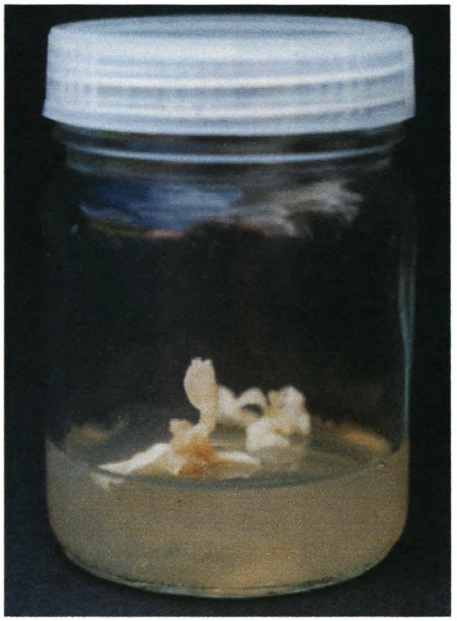

Fig. 4.5. Appearance of "negative control" shoots on MS medium containing the selective agent, Basta. As expected, after only a few days the "negative control" shoots died on Basta.

Fig. 4.6. Appearance of two "antisense" tobacco plants hardened off in sterile soil for further use in molecular analysis.

4.4 CONCLUDING REMARKS

Agrobacterium-mediated transformation has been used successfully for introducing useful genes such as the CP gene into a variety of crop species such as potato (Palucha *et al.*, 1998) and grapevine (Brault *et al.*, 1993). Expression of such a pathogen-derived gene in the transgenic plant can provide the plant with resistance to that pathogen (Sanford & Johnston, 1985). The two recombinant plant expression vectors, pCamBLR3CP+ and pCamBLR3CP-, which host the GLRaV-3 CP gene in the sense and antisense orientations respectively, were used to transform tobacco via *Agrobacterium*-mediated transformation. Each of these vectors contains the *bar* herbicide resistance gene, which gives a plant expressing the *bar* gene the ability to detoxify PPT. Phosphinothricin is the active ingredient in Basta and inhibits glutamine synthase activity, which is ultimately responsible for ammonium assimilation and nitrogen metabolism in plants. Plants that do not express the *bar* gene will therefore die due to the accumulation of ammonium (De Block *et al.*, 1987).

Due to contamination problems, only a few of the leaf discs were regenerated into full-grown tobacco plants. Of these leaf discs, those that were transformed with pCamBLR3CP+, pCamBLR3CP- or pCAMBIA 3301 survived on plant tissue culture medium containing Basta. The regenerated plants were treated with an antifungal solution, Captan, which was kindly donated by M. Vivier.

In conclusion, *Nicotiana tabacum* L.cv.'Xanthi' leaf discs were transformed with avirulent *Agrobacterium* harboring either the pCamBLR3CP+, pCamBLR3CP- or pCAMBIA 3301 binary vectors. Tobacco plants were regenerated for use in molecular analysis.

CHAPTER 5

THE MOLECULAR ANALYSIS OF PUTATIVE TRANSGENIC *NICOTIANA TABACUM* PLANTS

SUMMARY

The tobacco plants putatively transformed with pCamBLR3CP+ and pCamBLR3CP- were screened for the presence of the GLRaV-3 CP gene. PCR with GLRaV-3 CP gene specific primers showed no amplification of the GLRaV-3 CP gene in all the surviving "sense" and "antisense" plants. Southern blot analysis with the GLRaV-3 CP gene as hybridization probe showed no signal for these plants, thus confirming the PCR results. The "positive control" tobacco plants putatively transformed with pCAMBIA 3301 were screened for the presence of the *bar* gene by using PCR and *bar* gene specific primers. These plants showed no amplification of the *bar* gene. The "sense" and "antisense" plants were also screened for the presence of the *bar* gene and three plants showed amplification of the ~560 bp *bar* gene. This result suggests that these three plants were transformed with either pCAMBIA 3301 or a portion of the T-DNA region of pCamBLR3CP+ or pCamBLR3CP-. The latter could be the result of partial T-DNA integration into the tobacco genome. This concludes that the transformation procedure was for the most part unsuccessful.

5.1 INTRODUCTION

With the dawn of the biotechnology era came the molecular tools to rapidly screen putative transgenic plants for the integration and expression of the transgene. The expression of a pea gene in transformed petunia callus was one of the first experiments to show that introduced genes can be expressed in transformed cells (Broglie *et al.*, 1984). The simplest method to screen for the incorporation of the transgene is by DNA amplification using PCR (Mullis & Faloona, 1987). Due to the persistent nature of *Agrobacterium*, selecting on antibiotics or herbicides alone can result in false positives. PCR therefore confirms the presence of the transgene in the plant genome. The next step is to confirm the integration and copy number of the transgene in the plant genome. This is achieved by performing a Southern blot (Southern, 1975). The ideal is to have single copy inserts, so as to prevent gene silencing (Wassenegger & Pélissier, 1998). Once the presence and integration of the transgene has been confirmed, the next step is to test for the expression thereof on a transcriptional level. This is achieved by performing a Northern blot (Alwine *et al.*, 1977). Finally, a Western blot is used to test for the expression of the transgene protein (Towbin *et al.*, 1979).

The expression of pathogen-derived genes in transgenic plants can give rise to PDR (Sanford & Johnston, 1985). There have been numerous reports where expression of the CP gene confers resistance in the form of CPMR (reviewed in Hackland *et al.*, 1994). This form of resistance is due to an accumulation of protein. The expression of either sense or antisense CP RNA can also be sufficient to provide protection. This form of resistance only requires the accumulation of nucleic acid, for example, the antisense CP gene of PLRV conferred resistance to the virus (Palucha *et al.*, 1998) and an untranslatable CP gene RNA sequence was sufficient in providing resistance to PVY (Smith *et al.*, 1994).

Tobacco has previously been transformed with the CP gene of GLRaV-3 in the sense and antisense orientations (Ling *et al.*, 1997). The CP gene only in the sense orientation was, as expected, expressed. Unfortunately because

GLRaV-3 can not be transmitted to herbaceous species, resistance to the virus could not be evaluated. There are however transgenic grapevines, which have been transformed with the GLRaV-3 CP gene. These plants are still in the process of been evaluated for virus resistance (Ling, 2000).

This chapter reports the screening of tobacco plants putatively transformed with the CP gene of GLRaV-3 in the sense and antisense orientations respectively.

5.2 MATERIALS AND METHODS

5.2.1 *Genomic DNA extraction*

Genomic tobacco DNA was prepared from the 11 putative transformants and from untransformed tobacco plants according to the protocol described by McGarvey *et al.* (1991) with minor modifications. Approximately 40-50 mg of leaf tissue was ground in a microcentrifuge tube in 800 µl extraction buffer (1 M Tris-HCl, pH 8, 3 % CTAB, 1.4 M NaCl, 0.02 M EDTA) and a pinch of carborundum. The mixture was vortexed for 10 seconds and heated at 60 ºC for 1 hour. Eight hundred microliters of chloroform was added to the homogenate, the mixture was vortexed and then centrifuged at 10 000 g for 5 minutes. The aqueous phase was removed and 1/2 volume phenol and 1/2 volume chloroform:isoamylalcohol (24:1) was added, mixed well and centrifuged as before. The aqueous phase was removed again and 1 volume chloroform:isoamylalcohol (24:1) was added. The extract was mixed well and centrifuged as before. The aqueous phase was removed and the DNA precipitated with 1 volume isoproponol at –20 ºC for 1 hour. The DNA pellet was recovered by centrifugation at 12 500 g for 10 minutes and washed with 70 % ethanol, followed by a final centrifugation step. The pellet was air dried and resuspended in 20 µl sterile H_2O containing 20 µg/ml RNase A (Roche Biochemicals). Aliquots of the DNA samples were electrophoresed on a 0.8 % TAE agarose gel to confirm the quality and quantity of genomic DNA. For quantification, the DNA was compared to DNA quantitation standards (GibcoBRL, Life Technologies).

5.2.2 *PCR and Southern blot analysis*

PCR using the GLRaV-3 CP and *bar* gene specific primers was performed to verify the presence of the T-DNA region containing the GLRaV-3 CP gene in the tobacco nuclear DNA of the "sense" and "antisense" plants. Southern blot analysis was performed according to the protocol supplied by Roche Biochemicals. This was done to confirm these PCR results. PCR using the *bar* gene specific primers was performed on the "positive control" plants to verify the presence of the T-DNA region of pCAMBIA 3301. The genomic DNA extracted from each plant was digested with *Nco* I and *BstE* II and electrophoresed overnight at 40 V on a 0.8 % TAE agarose gel (10 µg/lane). Fragments were then blotted on a Hybond N+ membrane (Amersham Pharmacia Biotech). The GLRaV-3 CP gene was PCR labeled with digoxigenin (DIG) and used as a probe for hybridization. Hybridization signals were detected by autoradiography using anti-DIG and the substrate CDP-Star™ (Roche Biochemicals). Genomic DNA (10 µg) from untransformed tobacco was "spiked" with pCamBLR3CP+ for use as positive controls in the Southern blot. An amount of pCamBLR3CP+ corresponding to the equivalent of 1, 3 and 5 copies of the integrated GLRaV-3 CP gene in the tobacco genome, was added to the untransformed tobacco DNA (for calculations see Appendix C.1).

5.3 RESULTS AND DISCUSSION

5.3.1 *Genomic DNA extraction and restriction enzyme digests*

Genomic DNA was extracted from the 11 putative transformants and from untransformed tobacco plants. Aliquots of the DNA samples were checked on a gel (Fig. 5.1) and quantified with the aid of high Mr standards (results not shown). Approximately 10 µg of each sample (2 "sense", 6 "antisense" and 1 "positive control") was used in the RE digests. The REs, *Nco* I and *BstE* II were used to cut the GLRaV-3 CP gene from the T-DNA region had integration taken place. The "spiked" untransformed tobacco DNA (representing 1, 3 and 5 copies of the GLRaV-3 CP gene in the transgenic

tobacco genome) was also digested with *Nco* I and *BstE* II, as this would cut out the GLRaV-3 CP gene from the T-DNA region in the construct, pCamBLR3CP+. Digestions were incubated over a much longer period to ensure complete digestion of the DNA samples had taken place. The digests were electrophoresed overnight to separate the fragments for use in blotting (Fig. 5.2).

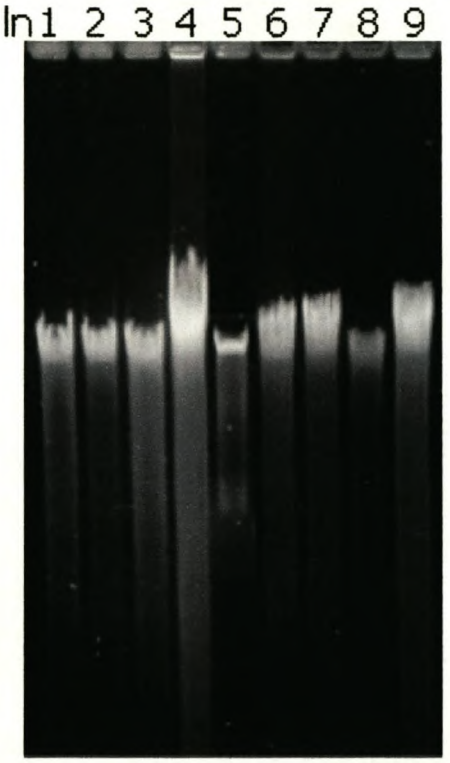

Fig. 5.1. Aliquots of genomic DNA extracted from the putative transformants on a 0.8 % TAE agarose gel. Lane 1 & 2, genomic DNA extracted from 2 "sense" plants. Lane 3-8, genomic DNA extracted from 6 "antisense" plants. Lane 9, genomic DNA extracted from 1 "positive control" plant. Aliquots of DNA from the untransformed plants were checked on a separate gel and appeared the same as the above DNA samples (results not shown).

Fig. 5.2. Separated fragments of tobacco DNA samples digested with *Nco* I and *BstE* II on a 0.8 % TAE agarose gel. Lanes 1, 2 & 3, digested untransformed tobacco DNA "spiked" with pCamBLR3CP+ so as to represent 1, 3 & 5 copies of the GLRaV-3 CP gene in the transgenic tobacco genome respectively. Lanes 4 & 5, digested genomic DNA from 2 "sense" plants. Lanes 6-10, digested genomic DNA from 5 "antisense" plants. Another gel that contained the digested genomic DNA of the 6th "antisense" plant and the "positive control" plant appeared the same as the above gel and was also used in the Southern Blot.

5.3.2 *PCR and southern blot analysis*

PCR results with the GLRaV-3 CP gene specific primers on the 2 "sense" and 6 "antisense" tobacco plants showed no amplification of the GLRaV-3 CP gene (Fig. 5.3). The PCR reactions were repeated a number of times using varying quantities of DNA, but the results were still negative. A positive control (pCamBLR3CP+) was included in the PCR, which yielded a clear amplification product of 975 bp, corresponding to the GLRaV-3 CP gene. As expected, however, the GLRaV-3 CP gene did not amplify from DNA of the 1 "positive control" plant, as it was only putatively transformed with pCAMBIA 3301.

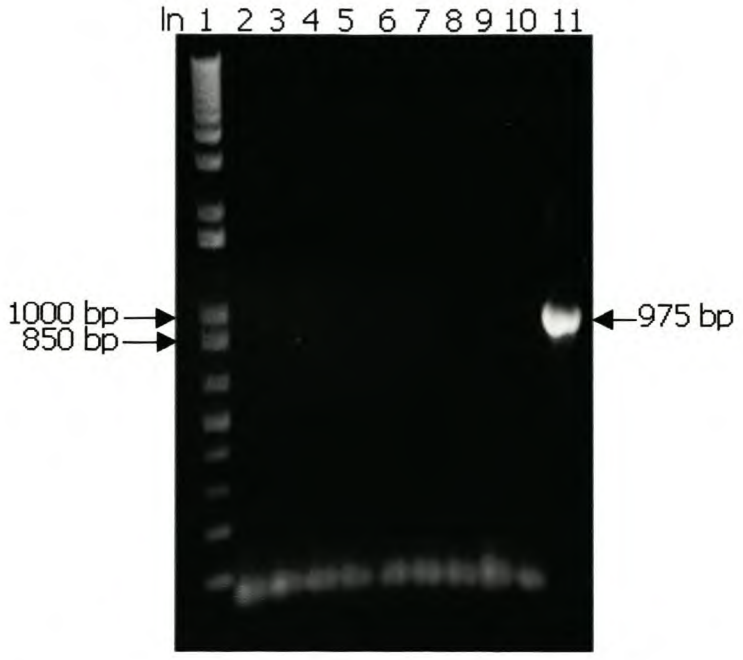

Fig. 5.3. Results of PCR performed on putatively transformed tobacco plants with the GLRaV-3 CP gene specific primers on a 1.4 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lanes 2 & 3, the GLRaV-3 CP gene was not amplified from the 2 "sense" plants. Lanes 4-9, likewise the GLRaV-3 CP gene was not amplified from the 6 "antisense" plants. Lane 10, as expected, there was no amplication product from the "positive control" plant. Lane 11, pCamBLR3CP+ was used in the PCR as a positive control and the amplified GLRaV-3 CP gene (975 bp) can be seen clearly on the gel.

The PCR results with the *bar* gene specific primers on the 2 "sense" and 6 "antisense" tobacco plants showed amplification of the ~560 bp *bar* gene from 3 plants but not from the "positive control" plant, which should have showed amplification of the *bar* gene (Fig. 5.4). This is a totally unexpected result, because one would assume that if the GLRaV-3 CP gene was not present in these 3 plants then the *bar* gene would also be absent. Once again a positive control (pCAMBIA 3301) was included in the PCR reaction which resulted in a clear amplification product corresponding to the *bar* gene. The *Agrobacterium* cultures used to infect the leaf discs were screened for the presence of the GLRaV-3 CP gene by PCR and proved to be positive (refer to Fig. 4.1). Although highly unlikely, the *Agrobacterium* cultures that were used for leaf disc infection could have been contaminated with pCAMBIA 3301. This

however, still does not explain why the plants would be transformed with pCAMBIA 3301 and not with pCamBLR3CP+ or pCamBLR3CP-. The fact that pCAMBIA 3301, pCamBLR3CP+ and pCamBLR3CP- all contain the bacterial Km resistance gene and the *bar* plant selectable marker gene, makes it difficult to eliminate pCAMBIA 3301 during antibiotic/herbicide selection steps. Although the *Agrobacterium* culture was grown from an individual colony which was PCR positive for the GLRaV-3 CP gene, the presence of a single bacterial cell containing pCAMBIA 3301 is all that is needed for extensive bacterial growth in the liquid LB and Km^{100} medium. What is also puzzling is why the rest of the "sense" and "antisense" plants were totally untransformed and yet still managed to survive on the selective agent, Basta?

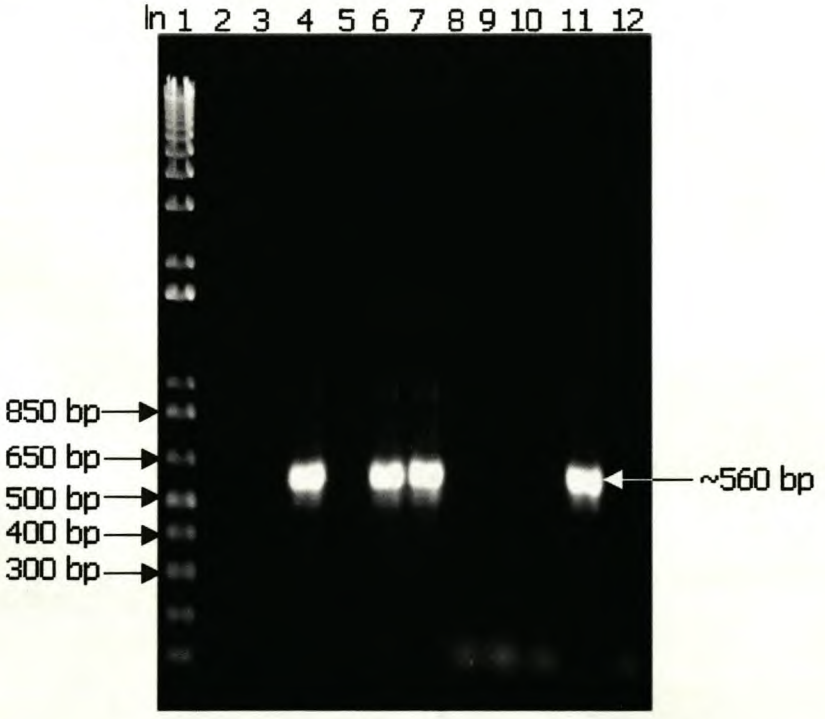

Fig. 5.4. Results of PCR performed on putatively transformed tobacco plants with the *bar* gene specific primers on a 1.4 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lanes 2, 3, 5, 8 & 9, the *bar* gene was not amplified from these 2 "sense" and 3 "antisense" plants. Lane 4, 6 & 7, the *bar* gene was amplified from these 3 "antisense" plants. Lane 10, the *bar* gene was also not amplified from the "positive control" plant. Lane 11, pCAMBIA 3301 was used in the PCR as a positive control and the amplified *bar* gene (~560 bp) can be seen clearly on the gel. Lane 12, H_2O was included in the PCR reaction as a negative control.

To confirm the negative PCR results, Southern blot analysis was performed on the 2 "sense" and 6 "antisense" tobacco plants using the PCR DIG-labeled GLRaV-3 CP gene as hybridization probe (Fig. 5.5).

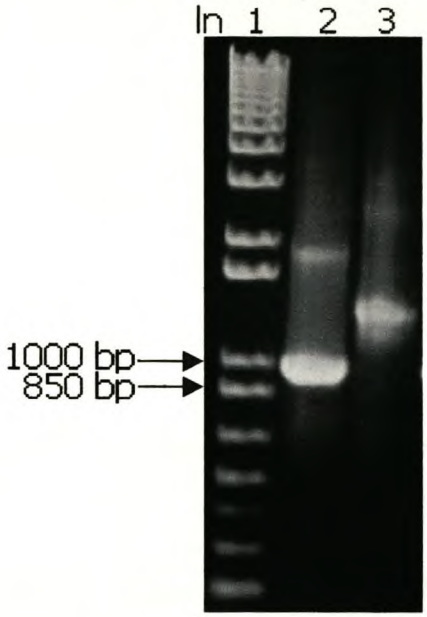

Fig. 5.5. PCR DIG-labeled hybridization probe (GLRaV-3 CP gene) on a 1.4 % TAE agarose gel. Lane 1, 1 kb plus DNA ladder. Lane 2, the unlabeled amplified GLRaV-3 CP gene is 975 bp. Lane 3, the labeled amplified GLRaV-3 CP gene appears bigger than 975 bp, due to the incorporation of the DIG deoxyuracil triphosphates.

As can be seen in Fig. 5.6, Southern analysis shows no hybridization of the GLRaV-3 CP gene to any of the 8 tobacco plants. The Southern blot positive controls, however, gave a clear hybridization signal representing 1, 3 and 5 copies of the GLRaV-3 CP gene in the genome of a transgenic tobacco plant. One would thus expect that the probe would be able to detect 1 copy of the GLRaV-3 CP gene in the tobacco genome.

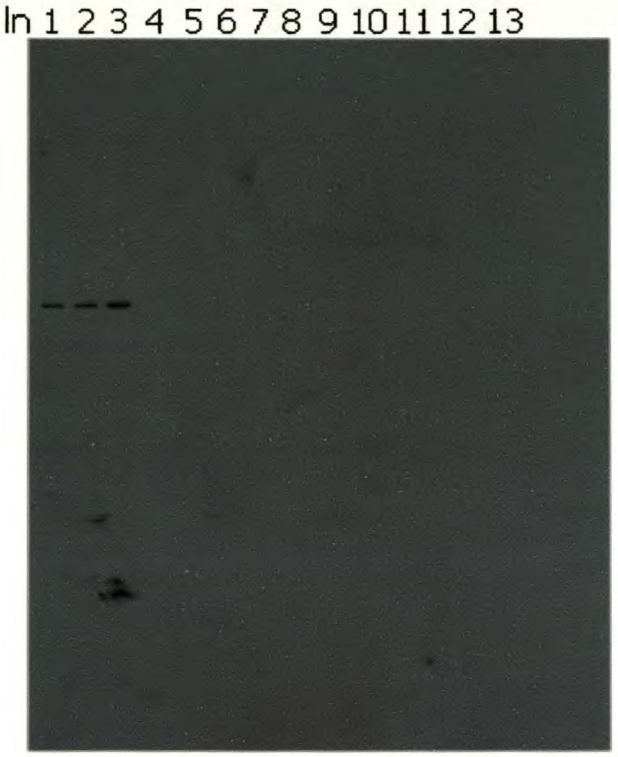

Fig. 5.6. Results of Southern blot analysis on putatively transformed tobacco plants using the PCR DIG-labeled GLRaV-3 CP gene as hybridization probe. Lanes 1, 2 & 3, hybridization of DIG-GLRaV-3 CP gene to the tobacco DNA "spiked" with pCamBLR3CP+ so as to represent 1, 3 & 5 copies of the GLRaV-3 CP gene in the transgenic tobacco genome respectively. Lanes 5 & 6, no hybridization signal for the 2 "sense" plants. Lanes 7-12, no hybridization signals for the 6 "antisense" plants. Lane 13, as expected, there was no hybridization signal for the "positive control" plant.

5.4 CONCLUDING REMARKS

The putative "sense" and "antisense" transgenic tobacco plants were screened for the presence of the GLRaV-3 CP gene by PCR. All the regenerated plants tested did not show amplification of the CP gene. These results were confirmed with Southern blot analysis. These plants were also tested for the presence of the *bar* gene by PCR. Three of the plants showed amplification of the *bar* gene. This result is very puzzling, since one would expect that if the plants did not show amplification of the CP gene, they would also not show amplification of the *bar* gene. After all, both genes are situated within the T-DNA region of pCamBLR3CP+ or pCamBLR3CP- and are

"theoretically" transferred as a unit to the plant cell genome. The only suggestions as to how this could have happened, although highly unlikely, are that the plants were either transformed with pCAMBIA 3301 or that due to partial integration, only the segment of the T-DNA region containing the *bar* gene of pCamBLR3CP+ or pCamBLR3CP- was integrated into the tobacco genome. The *Agrobacterium* cultures harboring either pCamBLR3CP+ or pCamBLR3CP-, which was used for infection, could have been contaminated with pCAMBIA 3301. This still does not explain why the plants infected with this "mixed" culture were not transformed with the pCamBLR3CP+ or pCamBLR3CP- binary vectors. Could these constucts be problematic for use in *Agrobacterium*-mediated transformations? Hopefully, the subsequent grapevine transformation experiments with these constructs will be able to answer this.

As mentioned before, all the "sense" and "antisense" plants were regenerated on plant tissue culture medium containing Basta. One would therefore also expect that if the plants survived on Basta, then they would contain the *bar* gene. However, the other five plants did not contain the *bar* gene, but yet still survived on Basta. The case of regenerating "false positives" resistant to Basta has recently been reported by Rasco-Gaunt *et al.* (1999).

The ultimate goal of transforming tobacco with the GLRaV-3 CP gene in the sense and antisense orientations was to test for CP expression.
Ling *et al.* (1997) has previously transformed tobacco with the GLRaV-3 CP gene in the sense and antisense orientations and as expected, only the CP gene in the sense orientation was expressed.

In conclusion, the transformation of tobacco with the recombinant plant expression vectors, pCamBLR3CP+ and pCamBLR3CP- was unsuccessful.

CHAPTER 6

GENERAL DISCUSSION

Grapevine leafroll is the most serious viral disease affecting South African grapevines. Symptoms include interveinal reddening in red grape cultivars and interveinal chlorosis in white grape cultivars, associated with a downward rolling of the leaf lamina. The disease affects the quality of the grapes by delaying maturation and lowering the sugar content of the berries (Krake, 1993). There are eight serologically distinct closteroviruses (GLRaV-1 to –8) associated with the disease (Boscia *et al.*, 1995, Choueiri *et al.*, 1996, Hu *et al.*, 1990a, Monis & Bestwick, 1997, Zimmerman *et al.*, 1990). Of these, GLRaV-1, -2, -3 & -7 are considered as genuine agents of GLR, whereas the other four are only associated with the disease (Boscia *et al.*, 1995, Grammatikaki & Avgelis, 2000). GLRaV-3 is considered the most economically important as it is spread rapidly in the field by mealybugs (Jordan, 1993, Cabalero & Segura, 1997, Petersen & Charles, 1997, Engelbrecht & Kasdorf, 1990). In South Africa, particularly, re-infection of healthy vines with GLRaV-3 is a very serious problem (Kriel, pers. comm.).

Virus control measures, which exist in the grapevine industry today, are to a large degree preventative measures and certainly do not provide any long-term solution. With the dawn of the biotechnological era came the tools and techniques with which to study organisms in a more detailed manner. Similarly, the interactions between host and pathogen could be investigated on a molecular level. Sanford & Johnston (1985) first proposed the concept of pathogen-derived resistance. As the name implies, resistance to a disease in transgenic plants is based on the expression of a pathogen-derived gene or genome fragment. The CP gene of TMV was used in the first demonstration of PDR in transgenic tobacco plants in the form of coat protein-mediated resistance (Powell-Abel *et al.*, 1986) and has since been applied to a number of crop species (Grumet, 1995). Subsequently, many other forms of PDR against plant viruses (and other pathogens) have been developed. These

include replicase-mediated resistance (Golemboski *et al.*, 1990), movement protein-mediated resistance (Lapidot *et al.*, 1993), RNA-mediated resistance (Prins & Goldbach, 1996) and antisense RNA-mediated resistance (Palucha *et al.*, 1998). Currently, CPMR has proved to be the most effective and certainly provides an attractive possibility for introducing virus resistance into crop species where no naturally occurring resistance genes have been identified, such as in grapevine (Gölles *et al.*, 2000). A number of transformation experiments involving important grapevine viruses, including GLRaV-3 are currently underway (Gonsalves, 2000, Minafra *et al.*, 1998, Xue *et al.*, 1999). In fact, tobacco plants expressing the CP gene of the GFLV were totally resistant to the virus (Gölles *et al.*, 2000).

The phloem-bound nature of GLRaVs and the occurrence of mixed infections in grapevine have made purifying pure virus isolates almost an impossible task (Choueiri *et al.*, 1996). The extraction and analysis of dsRNA proved to be an alternative method in detecting closteroviruses from leafroll infected vines (Mossop *et al.*, 1985, Rezaian *et al.*, 1991, Habili & Rezaian, 1995). A few research groups abroad have since cloned parts of the ~18 kb dsRNA associated with GLRaV-3 infected vines, but were unable to prove that these clones represent part of the GLRaV-3 genome (Habili *et al.*, 1995, Salderelli *et al.*, 1994). In 1997, Ling *et al.*, immunoscreened a cDNA library, that was prepared from the ~18 kb dsRNA, with GLRaV-3 specific polyclonal and monoclonal antibodies. They isolated, cloned and sequenced the CP gene of GLRaV-3.

We in collaboration with the grapevine transformation team at the Institute for Wine Biotechnology, Stellenbosch, decided to target the CP gene of GLRaV-3, in the hope of producing resistant grapevine cultivars. The initial step was to isolate and clone the GLRaV-3 CP gene into suitable plant expression vectors. The grapevine transformation team would then use these constructs for transforming grapevine.

In conclusion: This project focused on the isolation and cloning of the CP gene of a South African isolate of GLRaV-3 and to explore the possibility of introducing GLR resistance in South African vineyards.

A dsRNA of ~18 kb was extracted from South African GLRaV-3 infected material and used as a template for the production of cDNA. The cDNA was used as a template and together with GLRaV-3 CP gene specific primers, was used to amplify the CP gene of GLRaV-3 by PCR. The CP gene was cloned into a plasmid and sequenced. The data obtained from sequencing revealed a 99.26 % similarity to the only other GLRaV-3 CP gene sequence (Ling *et al.*, 1997). Two clones were selected by RE size analysis: one with the CP gene in the sense orientation (pLR3CP+) and one with the CP gene in the antisense orientation (pLR3CP-). The CP gene was excised from pLR3CP+ and pLR3CP- and subcloned into the plant expression vector, pCAMBIA 3301 in the sense and antisense orientations respectively. The CP gene was also subcloned in the sense orientation into another plant expression vector, pCAMBIA 2301. The recombinant pCAMBIA 3301 vectors hosting the GLRaV-3 CP gene (sense and antisense) was used for transforming tobacco to test for expression of CP. Unfortunately the tobacco transformation experiment failed. We have subsequently been informed that grapevine has been transformed with these constructs and are in the regeneration phase (Vivier, pers. comm.). I believe that the construction of suitable plant expression vectors for transforming grapevine was of utmost importance and that it contributed substantially towards "solving the virus problem". Future work will focus on evaluating resistance, if any, in grapevine to determine if the resistance is CPMR, RNA-mediated resistance or antisense RNA-mediated resistance.

APPENDIX A

A.1. *PCR specifications*

Amplification of the GLRaV-3 CP gene was performed by using the GLRaV-3 CP gene specific primers, LR3-CP02-For and LR3-CP02-Rev.

LR3-CP02-For: GATCTAGACCATGGCATTTGAACTGAAA
LR3-CP02-Rev: GTTCTAGAGGTCACCGATCGTAGCTACTT

The final concentrations of the PCR reagents and the thermocycler conditions are given below. In each PCR performed, H_2O was included as a negative control. The reaction mixes were aliquoted in 0.2 ml Eppendorf tubes and dH_2O added to a final reaction volume of 25 µl. GibcoBRL, Life Technologies manufactured the primers. Biotaq™ DNA polymerase, Mg^{2+} free 10x NH_4^+ buffer, $MgCl_2$ and dNTP's were supplied by Bioline. PCR products were electrophoresed on a 1.4 % TAE agarose gel and a 1 kb plus DNA ladder (GibcoBRL, Life Technologies) was used to determine the size of the fragments.

Reagents	*Final concentration*
NH_4^+ buffer	1x
$MgCl_2$	1.5 mM
dNTP's	200 µM
Taq polymerase	1.25 U/rxn
LR3-CP02-For	0.5 µM
LR3-CP02-Rev	0.5 µM
Template DNA	~50 ng
ddH_2O	variable

The following PCR cycle conditions were used to amplify the GLRaV-3 CP gene. An initial low stringency annealing phase was used to ensure incorporation of the 5' and 3' RE extensions in the PCR product. The annealing temperature of the high stringency phase was calculated using the Primer Calculator: nearest neighbour method. All reactions were performed using the Perkin Elmer 2700 thermocycler.

Phase	*Temperature*	*Time*	*No. of cycles*
Denaturation	94 °C	5 min	1
Denaturation	94 °C	30 sec	
Annealing	37 °C	45 sec	5
Extension	72 °C	60 sec	
Denaturation	94 °C	30 sec	
Annealing	60 °C	45 sec	30
Extension	72 °C	60 sec	
Extension	72 °C	7 min	1
Hold	4 °C	indefinitely	-

A.2. *Restriction enzyme digests*

Restriction enzymes were obtained from Promega and Roche Biochemicals. The incubation buffers provided with the RE was used throughout; for digestions with *Pml* I, *Apa* I, *Sma* I and *Bgl* II, bovine serum albumin was added to the incubation buffer to a final concentration of 100 µg/ml. All digestions were incubated at 37 ºC for 1-2 hours, except for *Sma* I and *BstE* II, which were incubated at 25 ºC and 60 ºC respectively.

Approximately 1-5 µg DNA was digested using 1 U restriction enzyme per µg DNA in a 20 µl reaction. Occasionally multiple digests were done, simultaneously if both the incubation buffer and temperature were compatible, and sequentially if the buffer and temperature differed (starting with the enzyme with the lowest salt concentration; see below). If the buffers were extremely incompatible, the DNA was phenol/chloroform extracted and ethanol precipitated between digests.

Small aliquots of digested DNA were checked for complete digestion by electrophoresis on a 1 % TAE agarose gel.

Below is an example of a multiple digest with *Nco* I and *BstE* II, starting with *BstE* II and supplementing the digest with Tris-HCl, pH 7.5 before digesting with *Nco* I.

Amount	*Reagent*
10 µl (5 µg)	DNA
2 µl	Buffer D (6 mM Tris-HCl, 6mM $MgCl_2$, 150 mM NaCl, pH 7.9)
0.5 µl (5 U)	*BstE* II
0.2 µl (1/10 dilution of 100 mg/ml)	bovine serum albumin
7.3 µl	ddH_2O
20 µl	Total volume
Incubate at 60 °C for 1.5 hours and then add the following:	
1 µl	Tris-HCl, pH 7.5
1 µl	Buffer H (90 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5)
1 µl	*Nco* I
8 µl	ddH_2O
30 µl	Final volume
Incubate at 37 °C for 1.5 hours	

A.3. *Ligation of vector and insert DNA*

All ligation reactions (except for those with the pGem®-T Easy vector system) between vector and insert DNA were done with the rapid ligation kit from Roche Biochemicals. Typically, 50-100 ng of vector DNA was used, with the total amount of insert and vector DNA to be ligated not exceeding 200 ng in a 21 µl reaction volume (see below for formula). An example of a typical ligation reaction between insert and vector DNA is given below. Only 1-2 µl of the ligation reaction mix was used directly afterwards for the transformation of *E. coli* strain DH5α competent cells. In cases where the transformation had to be done the following day, the mix was stored at 4 °C.

This formula was used to calculate the amount of insert DNA (ng) to be used for a desired insert : vector ratio in a ligation reaction:

$$\frac{\text{ng of vector x size of insert (kb)}}{\text{size of vector (kb)}} \times \text{insert : vector ratio} = \text{ng of insert}$$

This is an example of one of the ligation reactions that was performed: the concentration of the vector DNA was 209 ng/µl and the insert DNA was 24 ng/µl. The amount of vector DNA used was 50 ng in a 5:1 insert to vector ratio. The size of the vector was 9,258 kb and the size of the insert was 0,952 kb.

Therefore: $\frac{50 \text{ ng of vector x } 0.952 \text{ kb}}{9.258 \text{ kb}} \times 5 \text{ insert } / 1 \text{ vector} = 25.7 \text{ ng insert}$

Reagent	*Amount*
Vector DNA	2.39 µl of a 1/10 dilution
Insert DNA	1.07 µl
ddH_2O	4.54 µl
5x dilution buffer	2 µl
2x ligase buffer	10 µl
Ligase enzyme	1 µl
Final volume	21 µl
Incubate for 5 min at room temperature	

APPENDIX B

B.1. *Nucleotide sequence of the 942 bp CP gene of a South African isolate of GLRaV-3: sequenced from pLR3CP+ and pLR3CP-*

The bold red nts indicate the differences between the South African GLRaV-3 CP gene sequence and the published sequence by Ling *et al.* (1997).

5'ATGGCATTTGAACTGAAATTAGGGCAGATATATGAAGTCGTCCCCGAAAATA
G
ATTTGAGAGTTAGAGTAGGGGATGCGGCACAAGGAAAATTTAGTAAGGCGA
GTTTCTTAAAGTACGTTAAGGACGGGACACAGGCGGAATTAACGGGAATCG
CCGTAGTGCCCGAAAAATACGTATTCGCCACAGCAGCTTTGGCTACAGCGG
C
CGCAGGAGCCACCTAGGCAGCCACCAGCGCAAGTGGTGGAACCACAGGAA
ACCGATATAGGGGTAGTGCCGGAATCTGAGACTCTCACACCAAATAAGTTG
GTTTTCGAGAAAGATCCAGACAAGTTCTTGAAGACTATGGGCAAGGGAATA
G
GCTTTGGACTTGACGGGAGTTACCCACAAACCGAAAGTTATTAACGAGCCA
C
GGGAAAGTATCAGTAGAGGTGGCAATGAAGATTAATGCTGCATTGATGGAG
G
CTGTGTAAGAAGGTTATGGGCGCCGATGACGCAGCAACTAAGACAAAATTC
TTCTTGTACGTGATGCAGATTGCTTGCACGTTCTTTACATCGTCTTCGACG
GAGTTCAAAGAGTTTGACTACATAGAAACCGATGATGGAAAGAAGATATATG
CGGTGTGGGTATATGATTGCATTAAACAAGCTGCTGCTTCGACGGGTTATG
AAAACCCGGTAAGGCAGTATCTAGCGTACTTCACACCAACCTTCATCACGG
CGACCCTGAATGGTAAACTAGTGATGAACGAGAAGGTTATGGCACAGCATG
GAGTACCACCGAAATTCTTTCCGTACACGATAGACTGCGTTCGTCCGACGT
T
ACGATCTGTTCAACAACGACGCAATACTAGCATGGAATTTAGCTAGACAGC
T
AGGCGTTTAGAAACAAGACGGTAACGGCCGACAACACCTTACACAACGTCT
TCCAACTATTGCAAAAGAAGTAG3'

APPENDIX C

C.1. *Calculations for Southern blot positive controls*

The positive control conditions for the Southern blot has to be as close to what it would be like in the tobacco genome, therefore one can assume that:

- 1 molecule of pCamBLR3CP+ = 1 copy of the CP gene in the tobacco genome
- and the tobacco genome = 4.8×10^9 bp, and pCamBLR3CP+ = 10.21×10^3 bp
- so pCamBLR3CP+ : tobacco genome = 10.21×10^3 bp : 4.8×10^9 bp, therefore the ratio is 2.13×10^{-6} : 1
- for the Southern blot, 10 µg (10×10^{-6} g) of tobacco DNA was used for the digestions
- therefore, 2.13×10^{-6} **x** 10×10^{-6} g = 2.13×10^{-11}
- and $2.13 \times 10^{-11} = 21.3 \times 10^{-12}$
- and 21.3×10^{-12} = 21.3 pg
- therefore 21.3 pg of pCamBLR3CP+ = 1 copy of the GLRaV-3 CP gene in the tobacco genome
- and 63.9 pg of pCamBLR3CP+ = 3 copies of the GLRaV-3 CP gene in the tobacco genome
- and 106.5 pg of pCamBLR3CP+ = 5 copies of the GLRaV-3 CP gene in the tobacco genome

- therefore the positive controls for the Southern blot are: 3 eppendorf tubes each containing 10 µg of untransformed tobacco DNA "spiked" with 21.3 pg, 63.9 pg and 106.5 pg of pCamBLR3CP+ respectively. The 3 tubes of 21.3 pg, 63.9 pg and 106.5 pg of pCamBLR3CP+, represent the equivalent of 1, 3 and 5 copies respectively of the GLRaV-3 CP gene in the tobacco genome

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